

Keep an eye on genetic screening

The potential abuses of genetic screening should not be exaggerated, but neither should they be ignored. Governments must ensure that private screening services operate responsibly. Careful oversight, even in a climate of deregulation, is essential.

ONE of the most striking aspects of modern genetics is the way in which the public controversies it generates rapidly become a reflection of broader concerns, in the process magnifying these concerns into caricature. This has long been the case with the ethics of genetic engineering itself, whose goals are frequently reduced — in the public's mind at least — to images of Frankenstein-like monsters. It is also a danger in current debates about the responsible use of genetic testing.

The issues involved are far from straightforward, ranging from the statistical uncertainty of the science of genome analysis, through the highly personal evaluation of risk, to difficult choices about the appropriate forms of medical intervention. For example, the genetic mutations associated with breast cancer — to take the most recent example of what is likely to be an increasing list of polygenic diseases for which screening becomes available — can create enormous complexities. Even where a mutation can be identified, translating this into information about the susceptibility of an individual to the disease can be daunting, given the other factors involved. So, too, can the choice of preventive measures, none of which has been shown to be fully effective.

The debate becomes even more complex when commercial factors come into play. Last month, for example, Myriad Genetics Inc. of Salt Lake City, Utah, announced that it was offering to carry out comprehensive sequence analysis of the recently discovered *BRCA1* and *BRCA2* genes for susceptibility to breast and ovarian cancer. The cost of the test is \$2,400, with \$395 for each additional member of an at-risk family once a mutation has been identified. For some individuals, particularly those with a family history of breast cancer, or coming from a social group (such as the Ashkenazi Jews) in which mutations in either gene are particularly prevalent, the test may well be worth the price. For others, the balance may be more difficult to judge, for example when the uncertainty created by the result of a screening test is no less worrying than the uncertainty caused by a lack of knowledge.

It is obviously essential that the information and advice given to women about whether to take the test, and how to interpret its results, are handled sensitively. Myriad has, to its credit, shown itself aware of this responsibility. For example, it has set up a scheme with the Dana-Farber Cancer Institute in Boston to provide a confidential registry of women who have been tested for *BRCA1* and *BRCA2* mutations, and has worked with genetic counsellors to draw up a national directory of counselling resources.

At the same time, however, there has been an almost messianic quality in the public statements of some of Myriad's senior scientists about the extent to which genetic screening is opening up a new world of medicine, in which the treatment of disease will be replaced by prevention — a distant and uncertain prospect than runs the risk of raising false hopes. And there is always a concern that the promise of fat dividends for shareholders is a major driving force behind any company's involvement in the health field.

What should the US government do? This issue is currently being examined by a joint working group established by the National Institutes of Health and the Department of Energy (see page 101). Some members of the working group are keen for the Food and Drug Administration (FDA) take on a regulatory role in ensuring that genetic screening is handled responsibly by private

companies. Others, particularly those associated the companies themselves, warn that zealous regulation is unnecessary, and will only increase costs.

The FDA has made clear its reluctance to take on this role. Its main argument is understandable — if morally questionable — namely that it lacks the resources to do the task properly, at least not without cutting its commitment to other tasks. But it would be a disgrace if an issue as important as the proper use of genetic testing was reduced to a struggle over money. Countries such as Britain, as committed as the United States to the principle of deregulation, have nevertheless acknowledged that there are areas in which more government-backed supervision, not less, is appropriate. Genetic screening is one of these areas, and the US working group should have the political courage to acknowledge it. □

Eurovision for science?

A newly conceived association for science and society in Europe deserves antenatal support.

RAISING the public profile of science, providing a forum for open debate about its impact on society, and helping to promote the professional interests of scientists in general: such are the purposes of the American Association for the Advancement of Science and equivalent bodies elsewhere. The aims are grandiose, and the organizations' achievements inevitably fall short of the visions of their founders. But media coverage of their annual meetings is sufficient to suggest that they continue to play a significant role.

Europe lacks an organization with such ambitions on a pan-European scale, whether within the member states of the European Union or more broadly. It has transregional bodies that represent the interests of elite academics, the heads of funding agencies, individual scientific disciplines, and more. But an open association, intended to include both natural and social scientists, as well as others with a direct interest in the sciences, should — with a well-publicized debate here or a critical report there — have a complementary role to play. Certainly, public debates at the national level within Europe about science-related issues of multinational dimensions are too often blinkered by a lack of appreciation of the interests and instincts of other countries.

For those reasons, *Nature* welcomes the fact that a body called Euroscience, which is intended to help fill that gap, is now being formed. The appearance of the first announcement to that effect in our pages (page 108) reflects that welcome. (There is no other link between 'Euroscience' and this publication.) The new organization will face many obstacles, Europe being the diverse, contentious and politically heterogeneous region that it is. And Euroscience has yet to take effective action to meet its vision.

But it is easy to snipe at visionaries. Better to give support at this embryonic stage to ensure that Euroscience's agenda is practicable, its goals clearly defined and attainable, and its targets likely to achieve maximum impact. Each is essential if the association is to be helped over its next practical hurdle: attaining financial support, and thus a clout that goodwill alone cannot provide. □



Acceptance of marijuana therapy prompts call for more research

San Francisco. In an unprecedented move, voters in California and Arizona last week passed laws approving the medical use of marijuana. Clinical research on the drug's usefulness for treating pain, nausea and loss of appetite in cancer, AIDS and other diseases has often been held up in the past for what many claim are political reasons.

Opponents of change in the laws had pointed out the lack of scientific evidence for therapeutic uses of the drug. But supporters seem to have won over voters with anecdotes of medical benefits, and the argument that anti-drug politics had repeatedly undermined efforts by scientists to quantify the drug's usefulness.

Indeed, the vote has stimulated a call for the US government to overcome its objections to research on the medical use of marijuana and to fund studies of its own.

The Arizona law is the broader of the two, allowing doctors to prescribe marijuana and other drugs designated as Schedule 1 — which includes heroin and LSD — if they can offer scientific evidence that the drug is medically useful. The California rule allows a patient or 'care-giver' to grow and possess marijuana if it has been recommended, either orally or in a written prescription, by a doctor to treat a particular illness.

The California Medical Association (CMA) called the votes a mandate for research into both the potential therapeutic effects of the drug and its potential dangers. The association urged the National Institutes of Health (NIH) to conduct controlled studies. "It's not a law enforcement question, it's a scientific question," says Steve Thompson, vice-president of government affairs for CMA in Sacramento.

But the only federal response so far has been a promise by the Drug Enforcement Administration that it will continue to treat the cultivation and possession of marijuana as a felony. The CMA has warned doctors that, given strong federal laws, the state vote is primarily symbolic, and that doctors may face prosecution if they prescribe the drug.

The association has long acknowledged that marijuana could be of significant benefit to patients. But Thompson says that anti-drug laws have made it impossible to carry out proper research. Meanwhile, he says, use among patients has become widespread.

The Federation of American Scientists has also called for research. But Mark Kleiman, a member of the federation's drug policy group, says he is not optimistic that the state laws will lead to an outburst of scientific activity.

"It's like being a flat-earther — it's not healthy for your career," says Kleiman, a policy analyst at the University of California in Los Angeles. Researchers have to seek assistance from the National Institute on Drug Abuse to obtain samples of the drug, and to overcome objections from drug enforcement agencies against marijuana as a drug of abuse.

One difficulty facing scientists carrying out experiments on marijuana is that they cannot be double-blinded, and dosage is difficult to measure and control. Donald Abrams, the most recent researcher to attempt a study, says he has encountered these obstacles and others in a four-year effort to win approval to analyse the effect of marijuana inhalation on AIDS wasting.

Abrams, who is assistant director of the AIDS programme at San Francisco General Hospital and professor of clinical medicine at the University of California, San Francisco, had won support for his trial from the Food and Drug Administration and from various advisory bodies in California.

But the NIH, whose support he needs to obtain the drug, last summer returned his proposal unrated. The NIH panel criticized

the study's design, and complained that toxic effects of the drug — including the potential development of atherosclerosis in AIDS patients if they absorbed too many calories after smoking pot — might be ignored.

Steve Schnittman, assistant director of clinical research in the Division of AIDS at the National Institute of Allergy and Infectious Disease, declines to comment on Abrams's study, but says that his institute is concerned about the potential danger in such studies from toxic contaminants such as fungi. "We welcome good science, we just haven't gotten it yet," Schnittman says.

Abrams says that the new laws make research on the medical use of marijuana essential. Because research on the beneficial effects of marijuana appears to be so unpalatable to government agencies, he is planning to resubmit his study proposal to NIH as an analysis of toxicity.

Abrams says that in the past he had believed that he would eventually be able to carry out his experiments if the science he wanted to pursue could survive the pressures of politics. "Now, the politics may promote the science," he says. "It's a topsy-turvy turnaround."

Sally Lehrman

Car-maker turns to cannabis — for fibre

London. **As California and Arizona last week legalized the medical use of cannabis (above), scientists at the Daimler-Benz car company have declared their own interest in the hemp plant *Cannabis sativa*. The researchers have found that fibre from the plant (see right) can be used instead of glass fibre to reinforce plastic components in vehicles.**

Manufacturers are beginning to replace fibre glass with natural alternatives, such as flax, because products containing fibre glass pose environmental problems during disposal. Recent German government legislation allowing cultivation of the fibrous hemp for industrial purposes has paved the way for research into using hemp fibres in place of fibre glass. The fibrous hemp holds only small quantities of the resin that contains what the company calls the "ignoble" drug.

**IMAGE
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"Hemp fibres have a number of advantages over flax," says a company statement. "They are more rigid than flax and can be cultivated without the use of insecticides. Initial investigations have shown that hemp matches and even surpasses flax in terms of performance potential and promises to be even more economical." □

Daimler-Benz Centre

CERN seeks bank loans to construct LHC

Munich. Member states of CERN, the European Laboratory for Particle Physics in Geneva, will almost certainly be asked next month to approve the raising of bank loans to allow the Large Hadron Collider to be built as planned by 2005.

The SFr2.6-billion (US\$2.06-billion) collider will be the world's most powerful particle accelerator. The project was thrown into confusion in July when Germany decided, for budgetary reasons, significantly to reduce its promised contribution for the next four years (see *Nature* 382, 285; 1996).

CERN's committee of council agreed in September that other member states should not be asked to make up Germany's shortfall. Instead, it proposed that these

countries should reduce their own contributions proportionately, and should ask CERN to make up the resulting 9 per cent annual shortfall partly by internal savings and partly by raising loans to extend the payment period by a further three years after the end of construction (see *Nature* 383, 370; 1996).

After several weeks of reflection, the committee agreed last week to stick to this recommendation. Germany has not moved from its position, and other financial options put forward, such as phasing in the German cuts more slowly, or trying to build the collider on a smaller budget, were rejected as unrealistic.

The committee stresses that it does not

want to consider a further option, that of building the collider in two phases over a longer period. That would make it unattractive to potential partners such as the United States, whose hoped-for participation in the project could help bring forward the completion date.

The new financing plan, with its reduced annual contributions from all 19 member states, is likely to prove acceptable to CERN's decision-making council meeting next month. In the United Kingdom it would release three-quarters of the additional £65 million (\$39.6 million) that scientists have been demanding for domestic particle physics research in the next decade.

Alison Abbott

Japan's tokamak success boosts its bid for reactor

Tokyo. Japan's upgraded tokamak fusion reactor, JT-60U, has achieved a long sought-after goal of reproducing conditions 'equivalent' to breakeven, where energy output equals energy input. This will strengthen Japan's bid to host the International Thermonuclear Experimental Reactor (ITER), if the machine, planned to be built by Europe, the United States, Russia and Japan, is ever realized.

The JT-60U, an upgraded version of the JT-60 tokamak that originally started operations in 1985, achieved its goal in experimental runs on 31 October and 1 November. The tokamak uses a deuterium-deuterium fuel, which has much lower reactivity than the deuterium-tritium fuel that is expected to be used in the first reactors for practical energy production, such as ITER. As a result, the experiments did not actually achieve breakeven.

Rather, researchers at the Japan Atomic Energy Research Institute (JAERI), which operates JT-60U, deduced that in the conditions attained in the experiments — an ion temperature of 1.9×10^8 K and an electron density of $9.7 \times 10^{19} \text{ m}^{-3}$ in the plasma during a confinement time of 0.97 seconds — the ratio of energy output to input would have been 1.05 in the case of a deuterium-tritium fuel. As such, the Japanese results represent the same level of achievement as those obtained more than four years ago at the Joint European Torus in the United Kingdom.

But the Japanese media are interpreting the results as a sign that Japan has made a significant step towards the realization of ITER. A press release from JAERI, for example, emphasizes that the experiments used a reversed shear mode of operation that Japan and some other ITER participants are proposing for ITER.

Fusion scientists point out that the confinement improvement achieved with the

reversed shear approach is only transient, and it will not be practical until — and if — it can be sustained. "I would welcome efforts by the JT-60U team in the coming years to verify this point," says Atsuo Iiyoshi, head of Japan's National Institute for Fusion Science, which, in a different approach, is building the world's largest helical fusion device.

Japanese government officials and industrialists do not hide the fact that they are keen to host ITER. Three locations are competing to be put forward as Japan's proposed site: the Mutsu Ogawara district in Aomori Prefecture on the northern tip of Japan's main island, close to where a huge nuclear fuel recycling and processing facility is being built; Tomakomai in the northern island of Hokkaido; and Naka in Ibaraki Prefecture, northeast of Tokyo, the site of part of JAERI and Japan's laboratory for the engineering design phase of ITER.

Keidanren, a powerful federation of Japanese companies, is "strongly" backing the idea of Japan hosting ITER, according to a spokesman. Japanese industry apparently sees opportunities for lucrative contracts for the construction of ITER — which will cost billions of dollars — and the opportunity to learn about this technology.

Japan is even said to have put forward an informal proposal to pay up to 70 per cent of the total costs of ITER, estimated at more than US\$10 billion, if it can host the demonstration reactor (see *Nature* 380, 655; 1996). An official of the Science and Technology Agency, which oversees Japan's ITER programme, says the report of a 70 per cent offer is "not correct". But a Keidanren spokesman says that even if Japan has to pay this proportion, it would be a "good idea" for the country to host ITER.

Optimism as the JT-60U achieves fusion 'breakeven'.

The future of ITER itself hangs in the balance, as the United States cuts back on fusion research, and Europe questions whether magnetic confinement is the best approach to fusion (see *Nature* 380, 655; 1996). Japan's support could thus be critical.

There is disagreement, however, in Japan's fusion science community as to whether Japan should propose to host ITER at this stage. Iiyoshi says that he personally feels that the founding principle of equal sharing among ITER partners seems to be "unravelling" and that the project needs to be "fundamentally restructured" before future research work and the location can be discussed.

"The current ITER design represents a compromise among sometimes conflicting demands from the participants," says Iiyoshi, emphasizing that success is being achieved at the expense of innovation. He complains that this design philosophy "has resulted in a costly behemoth that has shied away from innovative but riskier features", and concludes that the machine has become "too predictable" to "ignite intellectual enthusiasm". But such views are unlikely to deter powerful supporters of ITER in Japan.

David Swinbanks

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JAERI

Election outcome signals few policy shifts

Washington. Some new faces — but few new ideas — are expected to mould US science policy during the next two months as the White House and the Congress reshuffle their teams after last week's elections.

The voting brought no change in the parties in power in either branch of government. President Bill Clinton did not ask his entire cabinet to resign, as other second-term presidents have done to avoid the embarrassment of sacking individuals. But several resignations have been announced or are expected, starting last week with that of William Perry, the defence secretary.

Perry could well be replaced by John Deutch, a chemist and former provost of the Massachusetts Institute of Technology, who is currently director of the Central Intelligence Agency. Universities believe that Deutch's appointment would boost basic research support from the defence department.

Hazel O'Leary, the energy secretary, is also expected to resign, and Bill Richardson, a Democrat congressman for New Mexico, is being widely tipped as her replacement. But Congress is likely to press strongly for the abolition of the department. There is even speculation that Clinton could agree to a proposal floated by members of the staff of Senator Pete Domenici (Republican, New Mexico) that the department should be turned into an independent agency.

Harold Varmus, director of the National

Institutes of Health (NIH), and Donna Shalala, the health secretary, are both expected to stay on. So are Neal Lane, director of the National Science Foundation (NSF), and Dan Goldin, administrator of the National Aeronautics and Space Administration (NASA).

Jack Gibbons will continue to serve as the president's science adviser, at least for a few



Stevens may chair the Senate appropriations committee, Brown returns and Sensenbrenner may take over science (left to right).

months until a successor is found if he decides to leave.

In Congress, James Sensenbrenner (Republican, Wisconsin) is expected to assume the chairmanship of the Science Committee in the House of Representatives, following the retirement of Robert Walker. Sensenbrenner's main interest is the oversight of NASA, and he has had a combative relationship with Goldin.

George Brown (Democrat, California) will return as the committee's senior minority member, after a tight race whose outcome was not announced for five days (see

Nature 383, 749; 1996).

Subcommittees of the House of Representatives appropriations committee can expect reshuffling to accommodate the retirement of John Myers (Republican, Indiana) and the ambitions of Joseph McDade (Republican, Pennsylvania), the most senior Republican appropriator, who was denied the chair of the full committee two years ago. But John Edward Porter (Republican, Illinois) is likely to retain his chairmanship of the appropriations subcommittee that funds the NIH.

Meanwhile in the Senate, the NIH's most important ally, Mark Hatfield (Republican, Oregon), is retiring. He will probably be replaced as chair of the full appropriations committee by Ted Stevens (Republican, Alaska).

Nancy Kassebaum (Republican, Kansas), chair of the Senate Labor and Human Resources Committee, which has authorization responsibility for the NIH, has retired. Larry Pressler (Republican, South Dakota), chair of the Senate Commerce, Science and Transportation Committee, which has authorization responsibilities for agencies such as NASA and NSF, was defeated last week.

The departure of these two moderates will mean less benign and more fiscally stringent Senate oversight for the respective agencies, although the names of their successors will not emerge until senators meet later this month. **Colin Macilwain**

Affirmative action curb 'will hit minority recruitment'

San Francisco. Companies in California that rely on state support of minority groups to increase the racial range from which they recruit engineers and scientists have been expressing concern at the approval by voters of a change in the law to prohibit affirmative action in government hiring, contracting and school admissions.

The change in the law — known as 'proposition 209' — was approved last week as part of the California elections by a margin of 54.3 to 45.7 per cent.

Opponents of the proposition have already filed several legal challenges, while civil rights leaders have urged the US Justice Department to help block the law, in particular by invoking civil rights legislation to withhold funds from the University of California.

But supporters of the proposition have also filed suits to ensure compliance. Among their targets is a rule followed by California Community Colleges that race must be a factor in hiring decisions.

Richard C. Atkinson, president of the

University of California, told faculty members and administrative staff last week that the university would comply with the law, but would also continue to emphasize racial diversity. Government officials say they are waiting for a court ruling, although the law took effect immediately.

Gary Fazzino, manager of state government affairs for the computer company Hewlett Packard in Palo Alto, says the company has relied on programmes such as the Math, Engineering, Science Achievement (MESA) programme at the University of California to cultivate promising young scientists and engineers who might join its staff. MESA targets "disadvantaged and historically under-represented" high-school and college students and provides race-based peer groups, mentors and role models to spur them towards achievement.

"These programmes are essential," says Fazzino. He says such programmes have made a dramatic difference in the number of women and minorities interviewed by the

company at leading colleges. Hewlett Packard will maintain its commitment to affirmative action because it makes good business sense, says Fazzino.

Only one company, Pacific Gas & Electric Co., had taken a public position against proposition 209 before the election. Pete Wilson, the governor of California, publicly rebuked the company for its statements.

Some advocates have suggested that the private sector, including universities such as Stanford and the University of Southern California, should take responsibility for outreach programmes in the absence of government efforts.

Programmes such as MESA serve as a pipeline to deliver qualified students to science and engineering companies, says Mike Beasley, a software manager in Silicon Valley who is chairman of MESA's board. With affirmative action programmes shut down at the universities, there will be more need for such efforts, Beasley says.

Sally Lehrman

Space agency reform plans under scrutiny

Paris. France's Centre National d'Etudes Spatiales (CNES), the world's third biggest space agency and the driving force behind Europe's space programme, is on the verge of a major reorganization aimed at adapting to the new environment created by the end of the Cold War and the increasing commercialization of space activities.

But many observers have been disappointed by a draft of the agency's strategic plan for 1996 to 2006, which was presented last week at a large gathering of space administrators, industrialists and researchers in Paris. Critics claim that apart from the Ariane-5 programme — which is recognized as an "absolute priority" by CNES, the French government, and the European Space Agency (ESA) — the plan makes few distinctions as to the weight that needs to be given to CNES's various activities.

"Everything seems to be a priority [in the strategic plan]," says Mark Giget, head of the Paris-based space consultancy, Euroconsult. The plan "is very vague", adds one leading science administrator.

According to the critics, these failings suggest that CNES still has far to go before adapting to the painful changes demanded by the new political and economic climate. Francois Fillon, the French space minister, left delegates at last week's meeting in no doubt that the government wants to see CNES make such changes.

One reason the space agency is having difficulty in adapting is that it is only beginning to recover from a long-running internal crisis over its aims. One observer says this came to a head last year following the decision by Europe's space ministers at a meeting in Toulouse to agree on the participation of ESA in the international space station.

CNES has paid a heavy price for the agreement reached, and will need to make

economies of 5 per cent a year over the period 1996 to 2000 to pay for its contribution to the station programme. Investment in domestic programmes is likely to stagnate as a result.

The consultation carried out as part of the preparation of the strategic plan by Alain Bensoussan, CNES's new president, was a "psychoanalytical" exercise aimed at "remobilizing and convincing staff that CNES must adapt" to the new conditions imposed by the Toulouse agreements, says one observer.

Indeed, CNES is suffering from an identity crisis, according to observers, that stems from the agency having become a victim of its own success. It was created in 1961 to allow France to compete with the two superpowers, by laying the foundations of a French space industry.

But the industries that CNES helped create have matured, while the need to compete with the United States is prompting a spate of mergers between French aerospace companies and those in other ESA member states, leading to a slow but inevitable Europeanization of French and other national space industries. Fillon said France, with its large domestic industrial base in aerospace, should become the "pivot" of the inevitable consolidation of Europe's space industry.

This marks a shift in French policy, away from driving Europe's efforts in space via the CNES, towards achieving this by flexing competitive industrial muscle. Fillon made it clear last week that the government wants CNES — which currently operates 18 industrial subsidiaries in areas from Earth observation to telecommunications — to withdraw from direct support for mature space technologies.

More and more of the work once done by space agencies is now being carried out by

commercial companies. Fillon predicted that while the budgets of the world's space agencies would reach ECU150 billion (US\$119 billion) by the end of the century, the global space market would reach ECU360 billion.

"CNES needs to recentre its activities on long-term research and development," says Jean-Yves Le Gall, who is responsible for CNES's strategic plan. One longstanding



criticism of CNES is its relatively low spending on "advanced concepts" research such as robotics and artificial intelligence. "The job of CNES should be to identify technologies and develop advanced techniques" says one observer, adding that "industrial development should be left to industry".

Complicating matters further, the traditional role of space agencies in developing technologies is itself changing. One trend identified at last week's meeting was that technological innovations are increasingly originating from other sectors, such as computing, and the challenge for space agencies is now often to adapt these to the needs of space missions, as well as developing technologies from scratch.

CNES also faces an identity crisis in meeting the government's stated aim of making technological research and development more European. A European approach will become more and more important, said Fillon, arguing that this is essential if Europe is to build the critical mass needed to be a credible partner for the United States, Russia and Japan.

International collaboration will be the rule from now on, said Fillon, adding that European space policy needed to abandon its tradition of developing ambitious plans for an independent space programme. The Toulouse agreements had "modified the very sense of European space policy".

Fillon himself predicted last week that Europe's national space agencies would eventually be forced to rationalize their activities and become more European, although he admitted this would take a long time to come about.

Declan Butler

Bids open for supercomputing centres

Washington. The US Department of Energy is seeking bids from multidisciplinary teams in US universities eventually to host up to five centres that will help to develop supercomputer applications to support its nuclear weapons programme. But department officials deny reports that they are preparing to spend up to \$100 million a year in the near future on university research related to the weapons programme.

Universities will be asked this week to submit proposals to host one of three or four supercomputer centres that will be established during 1997 under the energy department's Advanced Strategic Computing Initiative (ASCI), a giant programme to simulate nuclear weapons

in the absence of testing. Dick Watson of the Lawrence Livermore National Laboratory in California says that the centres will have a joint budget of \$5 million in the first year, and that this will grow steadily to \$25 million five years from now.

According to Watson, the department also plans to spend between \$3 million and \$4 million a year on individual investigator research at the universities to support ASCI, and is considering widening university involvement in the weapons programme. But he dismisses as "absolutely crazy" a report last week that the weapons programme would spend up to \$100 million next year with the universities.

Colin MacIlwain

Scaled-down Cluster mission aims for relaunch in 1999

Munich. The Cluster satellite mission will be relaunched in 1999, provided that the European Space Agency (ESA)'s science programme committee approves a scaled-down plan, as expected, later this month. Cluster's original four satellites were destroyed when its Ariane-5 launcher exploded during its maiden voyage in June.

Last week, ESA's space science advisory committee voted unanimously to support a relaunch, despite the overall project cost of ECU210 million (US\$166 million) which the committee fears will have a significant impact on its long-term space science programme, Horizons 2000.

According to the committee, the costs of the relaunch could be met by reshuffling the financing of Horizons 2000 after 1998, postponing by six months the launch of the next cornerstone mission — a satellite dedicated to infrared and submillimetre astronomy — and delaying by two years a planned 'medium-sized mission' dedicated to Solar System science.

The new Cluster will be made up of the project's original spare satellite, Phoenix, and three smaller identical satellites, which will probably be built by German aerospace company Dornier. The four satellites are likely to be launched in two batches, one pair by Ariane-4 and the other by Ariane-5, at a cost that is expected to be kept down to ECU60 million.

According to Cluster's project manager, John Credland, most of the scientific aims of the mission will be saved. But ESA member states have so far been unable to produce enough money to replace all the instruments that were on the original four spacecraft.

Credland says there will even be a benefit from the delayed flight of Cluster. The mission will be able to analyse in three dimensions the Earth's electrical and magnetic fields as they interact with solar wind particles, as the satellites will benefit from maximal sunspot activity. This will occur in 2001 and 2002.

But the committee remains concerned that the relaunch could have a long-term impact on the Horizons 2000 programme, which plans space science missions for the next 20 years. The programme includes two further cornerstone missions — a mission to Mercury and an interferometry observatory — and a series of medium-sized missions. Also included are possible international collaborations in projects such as the international space station, the Hubble space telescope and its possible successor, the

Next Generation Space Telescope (NGST).

The committee will meet in January to 'brainstorm' the overall programme, in order to ensure that it is set on a reliable track once again. ESA prides itself on the stability of individual programmes that, thanks to long-term planning, protects it from the political uncertainties suffered by US space science programmes. But it fears this stability may be threatened because the financial reshuffling required by the Cluster relaunch leaves little financial buffer.

Also on the agenda of the January meeting will be ESA's participation in the Hubble space telescope after 2001. That year will see the expiry of a ten-year international agreement that recognizes ESA's contribution to Hubble's infrastructure and instruments. The agreement guarantees the agency's space scientists 15 per cent viewing time and 15 per cent participation in Hubble's scientific review panels.

A working party to negotiate a new agreement was set up last month by ESA and the US National Aeronautics and Space Administration (NASA). But this time ESA has

little bargaining power. Hubble is to be refurbished in 1997 and 1999 to extend its life to at least 2005. In 2002, during the final planned refurbishment, the original hardware contributed by ESA will be replaced by updated NASA hardware, and instruments will be added. Because of a shortage of money, ESA's science programme has been unable to compete to provide new instruments.

One controversial negotiating tool that the committee may consider is the promise of significant participation in NGST, a project that is as yet very vague both in design and financing. The European astronomical community is lobbying ESA to ensure continuing participation in Hubble and NGST.

Piero Benvenuti, head of the ESA Space Telescope European Coordinating Facility, in Garching near Munich, says that access to the space telescopes will be of particular importance when the European Southern Observatory's Very Large Telescope (VLT), now being built in northern Chile, comes into operation in 2000.

"Most of Hubble's biggest successes have been follow-ups to objects observed with the Keck's telescope in Hawaii," he says. "Space telescope follow-up by the VLT of objects identified with Hubble or the NGST will be of utmost importance for European astronomers."

Alison Abbott

Rocket failure leads to grounding of small US satellites

Washington. The US National Aeronautics and Space Administration (NASA) has suspended further launches of small satellites after a malfunctioning Pegasus rocket led to the loss of two scientific missions last week. Daniel Goldin, NASA administrator, called the moratorium until a newly formed review panel can assess the agency's options for putting small payloads safely into orbit.

Goldin has made no secret of his impatience with the US launch industry, particularly Orbital Sciences Corporation, the manufacturer of Pegasus, for a string of launch failures and delays that has been a stumbling-block for the agency's 'cheaper, faster' philosophy of small science missions (see *Nature* 381, 541; 1996).

Both the HETE (High Energy Transient Experiment) and the Argentine SAC-B science missions are believed to be total losses after the third stage of an air-launched Pegasus-XL rocket failed to separate from the satellites in orbit. SAC-B, which was primarily an engineering test of Argentina's first satellite, carried instruments to observe the cosmic X-ray background and hard X-rays from solar flares.

HETE was an international mission led by NASA and the Massachusetts Institute of Technology, with partners in France, Japan, and Italy. Its primary focus was on gamma-ray bursts; scientists had hoped it would be able to locate optical counterparts to these mysterious objects.

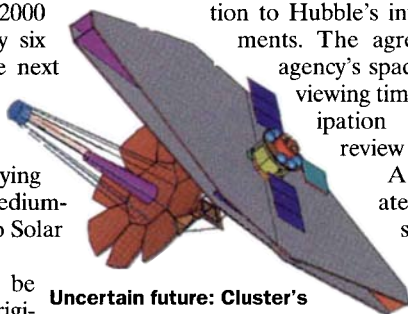
Although neither satellite was expensive by the standards of space missions, costing just over \$20 million each, their loss is "an embarrassment" admits Alan Bunner, who heads NASA's high-energy astrophysics programme. Goldin immediately ordered the agency's chief engineer, Daniel Mulville, to head a task force to consider the agency's options for launching small satellites.

Mulville says his team will consider how NASA managers can better assess risk and reliability, how much flexibility the agency has to switch launch vehicles under current contractual agreements, and whether it could expand its launch options to include non-US vehicles.

At present, only two US launchers are available for small satellites: Pegasus and the LMLV (Lockheed-Martin Launch Vehicle), which also failed in its first and only try last year.

Missions that could be affected by the moratorium in the short term are the SWAS (Submillimeter Wave Astronomy Explorer) scheduled for a Pegasus launch in January, and the Lewis and Clark satellites, the first of which is set for an LMLV launch on 20 December.

Tony Reichhardt



Uncertain future: Cluster's relaunch could affect the Next Generation Space Telescope.

Prediction sceptics 'must tackle bias' in earthquake research

London. Scientists who are sceptical that earthquakes can be predicted claim their views are being ignored and their research funding applications rejected because of bias by governments and the media.

Delegates at an international meeting of earthquake prediction sceptics in London last week said they were perplexed at the level of support for research into earthquake prediction, given that most predictions had failed. Many criticized what they considered unbalanced media coverage of earthquake prediction claims.

Russ Evans, head of regional geophysics at the British Geological Survey and one of the meeting's organizers, said studies that seek to reduce earthquake hazards are being underfunded because of prediction research. Fellow 'sceptics' should counter the public's tendency to believe in prediction claims, he said.

The two-day meeting was organized by the Royal Astronomical Society and the Association for Geophysics. Perhaps unsurprisingly, a consensus emerged that the influence of the earthquake prediction community is disproportionate to the scientific strength of its claims.

"If you give two proposals to a government, one of which says 'we predict an earthquake', the other that 'we want to do some basic research', guess which one gets the money," said Robert Geller, an associate professor at Tokyo University.

According to Geller, a century of earthquake prediction research has yielded little of significance beyond "specious claims of breakthroughs". Much of the blame, he said, lies with those who announce their claims without peer review.

Stathis Stiros, a researcher at the Institute of Geology and Mineral Exploration in Athens, said Greece's government is spending too much of the country's scarce research funding on earthquake prediction. "We should spend more on reinforcing our buildings."

But Bill Ellsworth, a geophysicist at the US Geological Survey in Menlo Park in California, says most earthquake research in the United States for the past decade has focused on the nature of earthquakes and on hazard reduction. "We don't do 'black box' predictions."

Ellsworth adds that researchers have made "enormous progress" over the past century. "We know where earthquakes occur. We understand more about the behaviour of complex fault systems over time and are making good progress towards long-term forecasts. This doesn't help you plan your weekend, but it helps reduce the risk to society." Ehsan Masood

Roche claims victory in bid for European *Taq* patent

London. For the second time in two years, the Swiss pharmaceutical company Hoffmann-la Roche says it has been told by the European Patent Office (EPO) that it is to be granted a broad European patent on thermostable enzymes used for the polymerase chain reaction (PCR) process.

According to company officials, the patent is "expected to be issued within the next six months". This follows a hearing at the EPO in Munich last week at which the company presented new evidence to counter claims that its discovery was not new, as the specific thermostable enzyme *Taq* DNA polymerase, obtained from the bacterium *Thermus aquaticus*, had previously been characterized by other researchers.

Two years ago, these claims were presented to the EPO examiners by rival companies, in particular the US laboratory equipment company Promega, which is currently being sued in the United States by Roche for infringing a licence to sell *Taq* polymerase. The patent office then suspended its previous decision to award the patent, saying that it wanted more information from Roche (see *Nature* 374, 108; 1995).

This summer, the examiners wrote to Roche saying that they would not be able to grant the patent unless they received satisfactory answers to key questions. They wanted more proof that the work carried out by David Gelfand and other scientists at Cetus, from which Roche bought the rights to *Taq* in 1991 as part of a US\$300-million package involving PCR technology, represented a significant step beyond those of previous researchers. Roche officials say that it was after they had produced such evidence at last week's meeting that the examiners agreed to issue the patent.

The EPO's decision gives Hoffmann-la Roche broad rights to a range of

thermostable enzymes with a molecular weight of between 86,000 and 90,000 daltons. According to Tom White, vice-president of research and development at Roche Molecular Systems in New Jersey, these include the DNA polymerases from *Thermococcus littoralis* (known as 'VENT'), and *Pyrococcus furiosus* ('Pfu'). As a result, the company has been given the right to royalties from producers of both enzymes.

White said last week that Roche welcomed the approval of the patent as "an important recognition of the biochemical facts that clearly distinguishes the invention of our scientists from prior work". The patent covers both natural and recombinant forms of thermostable DNA polymerases, and Roche officials say they have already negotiated licences with 18 suppliers.

The European decision is likely to be studied closely by attorneys for both Roche and Promega. In the US dispute, Promega has issued a counter-challenge to Roche, challenging the validity of the US patent and alleging that misleading information was deliberately used in the original patent legislation (see *Nature* 382, 660; 1996). A hearing of the case is due to be held in San Francisco on 13 December, having been delayed from last month at Roche's request.

Officials at Promega remain defiant. Randy Diamond, chief technical officer, said in a statement that "virtually the entire scientific community has acknowledged that any claim to native *Taq* DNA polymerase lacks novelty. Roche's latest statement does not dispute that basic premise". Roche, meanwhile, claims that Promega has "misrepresented the litigation and the science involved" and has "engaged in an aggressive public relations campaign to obscure the purely commercial nature of their interest in the PCR patents".

David Dickson

India commissions its thorium reactor

New Delhi. India's efforts to produce nuclear power from its supplies of thorium passed a significant milestone this month with the commissioning of a research reactor fuelled entirely by uranium-233 produced from thorium.

The Department of Atomic Energy (DAE) claims that the reactor, at the Indira Gandhi Centre for Atomic Research at Kalpakkam near Madras, is the only one in the world using such man-made material. It is also one of the smallest, using barely 600 grams of fuel to produce peak power of 30 kilowatts.

The reactor has been named Kamini,

short for 'Kalpakkam mini'. Rajagopala Chidambaram, secretary of the department, describes its successful commissioning as a "small but significant step towards the long-term goal of exploiting our thorium reserves".

India has 400,000 tonnes of thorium metal locked up in monazite-rich beach sands. Its energy content is equivalent to 500 billion tonnes of coal — sufficient to meet India's energy needs for several centuries. By itself thorium is not a nuclear fuel. But when exposed to neutrons it turns into fissile U-233, which can be separated by a chemical process.

K. S. Jayaraman

FDA resists regulatory role in gene tests

Washington. A government-sponsored task force is debating whether to recommend that the US Food and Drug Administration (FDA) take on a role in regulating genetic tests offered by specialized laboratories — even though the FDA itself has indicated a strong reluctance to do so.

The joint National Institutes of Health/Department of Energy Task Force on Genetic Testing may shortly decide to call for some degree of regulatory oversight of these genetic tests by the FDA, particularly those whose results need careful interpretation. This would be spelled out in draft recommendations that the task force plans to publish in January, before it makes final recommendations in March.

But the suggestion that the FDA should step into the controversial area is meeting resistance at the agency. FDA officials argue that they do not have the resources to regulate genetic tests, and that to do so would open the agency to having to take responsibility for regulating many other in-house, non-genetic tests currently offered by sophisticated 'reference' laboratories.

Neil Holtzman, director of genetics and public policy studies at the Johns Hopkins Medical Institutions, and chair of the task force, said last week that it is developing a classification scheme which ranks tests according to their level of "potential danger" to the public. It may ask the FDA to regulate those defined as most dangerous. Holtzman has expressed a personal belief that the growing field may not be manageable without some degree of regulation.

The task force is most concerned, says Holtzman, about tests in which a positive result does not imply a high probability that an individual will develop the disease in question; those in which a negative result does not imply a high probability that the individual will remain free from the disease; and those for mutations predisposing to diseases — such as Alzheimer's — for which there is no known or accepted treatment.

Holtzman's comments follow the commercial introduction late last month of full sequence testing of *BRCA1* and *BRCA2* genes by Myriad Genetics of Salt Lake City, Utah. Mutations in the two genes imply a strong predisposition to — but not the certainty of — breast and ovarian cancers in women with family histories of the disease.

The company is charging \$2,400 for initial testing, and \$395 for tests of additional family members. Testing is available only through doctors. The company has promised to provide full counselling on the implications of the results of its tests; but the move has rekindled the debate over whether the FDA should be given authority to judge whether such efforts are adequate.

Agency officials say this new responsibility would overtax their finite resources, forcing

them to cut back other commitments. At a task force meeting in September, Steve Gutman, a director of the FDA's Division of Clinical Laboratory Devices, protested that asking the agency to regulate genetic tests would be opening "an incredible universe" which would require "a new branch" to be handled properly. "What should we stop doing so we can do genetic tests?" he asked.

The agency's top official in the area agrees. "Should we be putting our resources into automated pap-smear readers [that affect millions of women every year], or into genetic tests that are available through a limited number of reference laboratories?" asks Bruce Burlington, director of the FDA's Center for Devices and Radiological Health.

In theory, genetic tests already fall under FDA regulation. The US Food, Drug and Cosmetic Act gives the FDA broad control over all *in-vitro* diagnostic products, including — according to the agency — tests

Care Financing Administration and the Centers for Disease Control and Prevention.

CLIA requires merely that in-house tests be 'analytically' valid, meaning that they accurately measure what they are meant to measure — in the case of genetic tests, that a given mutation is present. But, in contrast to the FDA's regulatory requirements, there is no standard under CLIA for 'clinical validity', which in the case of genetic tests would essentially boil down to the ability of a test to predict future disease.

According to Holtzman, the lack of such a requirement under CLIA has left the task force with "tremendous doubts" about the capability of the act to assure the quality of genetic tests.

The FDA disagrees. It has proposed a rule that acknowledges its regulatory authority over reagents used in in-house tests by specialized laboratories. But it has also said that it does not plan to regulate these reagents — including gene probes used in genetic tests — implying that such oversight would be left to CLIA.

The FDA has collected public comment on its proposed rule, and plans to offer a final version soon. Meanwhile, said Holtzman, companies marketing genetic tests as in-house services "say 'trust us'". So far, he adds, such companies have not shown themselves untrustworthy. "The problem is, can we be sure that other companies will be as responsible?"

Patricia Barr, an attorney who works with the National Breast Cancer Coalition and is a member of the task force, says that her group is "terribly concerned about clinical validation". It would like to see FDA approval tied to guarantees by companies that they will gather long-term data on patients who receive their tests.

But the companies are fighting to preserve the *status quo*. Patricia Murphy, vice-president of genetic services at OncorMed, told Congress this summer that the FDA is "not the appropriate agency to regulate genetic tests" conducted in reference laboratories. Myriad has warned that any FDA role "could stifle innovation and diffusion of genetic testing technologies".

Janet Haskell, president of Myriad Genetic Laboratories, the company's genetic testing facility, argues that, in the laboratory setting, "there's really no difference" between a blood cholesterol test and a genetic test. "No-one has given me a good reason why this test, or that type of test, would require FDA oversight, whereas others do not," she says.

The 15-member task force is expected to decide its position at a meeting early next month. Its eventual recommendations will be published for comment before being agreed in their final form next March.

Meredith Wadman

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

James King-Holme/SPL

Homing in: screening can reveal susceptibility to disease, but interpretation is uncertain.

administered as in-house services by specialized laboratories.

In practice, however, the agency has not regulated these tests, focusing instead on kits and reagents produced for sale, for example, to other labs and doctors' offices. No such kits are believed to exist currently for genetic tests. The FDA regulates the gamut of other kits, from home pregnancy test kits to cell counters used by hospitals and laboratories for standard blood tests.

The distinction between the FDA's treatment of kits and in-house tests has not been lost on companies such as Myriad, which has made *BRCA1* and *BRCA2* tests available only as in-house services. Similarly, Genzyme, of Cambridge, Massachusetts, offers in-house genetic testing for cystic fibrosis, and OncorMed, of Gaithersburg, Maryland, also tests in-house for mutations in *BRCA1* and *BRCA2* genes.

Under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, companies offering in-house laboratory tests as services — from the production of monoclonal antibodies that detect rare diseases to routine microbiological cultures — are monitored not by the FDA but by the Health

Vote on US breast cancer panel favours support for research

Washington. The steering committee of the US National Action Plan on Breast Cancer voted last week to return nearly all of its \$14.75 million 1997 budget to the National Cancer Institute (NCI).

The vote marks a dramatic assertion of power by those within the group who have argued that funds should be spent on advanced breast cancer research, and a defeat for those who want the action plan to play a more active role in areas such as educating physicians about breast cancer and encouraging studies of geographical clusters of its incidence. The action plan is a partnership between government agencies and breast cancer research advocates with a mandate to link activists and agencies in the fight against breast cancer. It was created in 1994, and placed in an office at the NCI, after pressure by activists led to support from President Bill Clinton.

Congress granted it \$14.75 million in 1996 and again in the 1997 fiscal year, which began on 1 October. But the steering committee voted last week by a 13:0 vote, with four abstentions, to recommend to Donna Shalala, the health secretary, that \$14 million of the 1997 allocation be returned "expeditiously" to the cancer institute, stipulating that it should be used for breast cancer research. □

Call for GMO food labelling

London. The UK government's Food Advisory Committee has reiterated its call for food manufacturers to provide information voluntarily for consumers if genetically modified organisms are used in their products. The call coincides with the end of a 24-hour protest by the environmentalist group Greenpeace against the importation of a mixed consignment of natural and genetically modified soya beans into the Belgian port of Antwerp from the United States.

Last week, more than 20 Greenpeace protesters chained themselves to the dock, preventing the gates from being opened. The protest was stopped on Friday afternoon when the US grain company Cargill, which has refused to segregate and label its shipment of genetically modified soya beans, obtained a court order that imposed a BFr1-million (US\$32,258) fine on Greenpeace for every extra hour the protest continued. □

Surveyor heads for Mars

Washington. The first of three international missions to Mars this year is under way, following the launch of the US Mars Global Surveyor (MGS) last week. The MGS will arrive in Mars orbit on 11 September next year to begin two years of systematic study of the planet's surface, atmosphere and magnetosphere.

One of the spacecraft's solar arrays did not deploy fully, but project managers expect the problem to be corrected. Russia's Mars '96 spacecraft is next up for launch on 16 November, and will arrive at Mars on 12 September next year with an orbiter, two surface landers and two penetrators. The US Mars Pathfinder will be launched on 2 December with a small lander and rover that will touch down on the martian surface on 4 July next year. □

Dog 'might have Gulf syndrome'

London. An Arizona veterinarian believes she may have come across a case of Gulf War syndrome in a dog that was in Saudi Arabia during the war. Valery Stephens of the Elmirage Animal Hospital in Surprise is treating an eight-year-old Doberman for immune-mediated thrombocytopenia, a condition in which the body destroys its own platelets, and other "bizarre neurologic signs" possibly related to the dog's travel history.

One of the dog's owners has been suffering from low platelet counts since returning from the Gulf, and is undergoing medical tests to establish whether she, too, has thrombocytopenia. Her partner, who left Saudi Arabia when he fell ill, died of cancer within

weeks of his return to the United States. Stephens says neither owners, nor the dog, have military connections. "We have no proof this is Gulf War syndrome," she emphasizes. "We're just looking for other cases, or possible answers." □

Genetical Society shuns China

London. Britain's Genetical Society has decided to suspend its affiliation to the International Genetics Federation (IGF), following controversy over the federation's decision to hold the next International Genetic Congress in Beijing, China, in 1998, despite the country's recent adoption of its 'eugenics' law. The decision by the society follows a ballot of its members, which produced a clear majority in favour of suspension (see *Nature* 383, 204; 1996). David Sherratt of the University of Oxford, the president of the society, said that despite the relatively low response to the postal ballot — only 7 per cent of the members returned ballot slips — the society's executive committee had taken the outcome as an expression of its members' wishes, and had voted virtually unanimously (with only one abstention) in favour of suspending its IGF membership. □

No-consent research rules

Washington. The US Food and Drug Administration (FDA) has approved new rules that would allow investigators in some cases to include patients in research without their consent. Medical researchers said the change allows the study of desperately needed new approaches to treating patients who are gravely ill with heart attacks, strokes or head injuries.

Under the new guidelines, patients can be used in research without their consent if the conditions are as follows: the patient has a life-threatening condition, the patient is unable to say whether he or she wants to participate, no relative is available to provide consent, the community in which the research is done has been notified of the study, and the research design has been approved by the FDA. □

Martian meteorites for sale

Boston. Three martian meteorite fragments will go on sale next week at Guernsey's, a New York City auction house. The rocks, which range in size from a golf ball to a grapefruit, are said to represent the only privately-owned collection that includes all known varieties of martian meteorites: Shergottite, Nakhilite and Chassignite.

The pieces could sell for about \$2 million in total, say Guernsey's, which is donating a portion of the proceeds to the American Cancer Society. In August the world's museum curators suspended the sale of meteorites from Mars, due to skyrocketing prices following the announcement by the National Aeronautics and Space Administration of evidence of possible microbial life on martian rocks. □

Industrialist to head CSIRO

Sydney. **Charles Allen (right), the recently retired chief executive of an Australian oil production company, has been appointed the part-time chairman of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), the country's main public research agency.**



His appointment reflects the desire of the new Coalition government to appoint more industrialists to head public bodies. Allen is the second chairman in CSIRO's 70-year history not to have been a professional researcher, although he brings 14 years' experience in exploration geophysics for the Shell Oil Company, mainly in West Africa.

CSIRO scientists have been concerned that the organization might be dismantled and partially privatized, as the Department of Scientific and Industrial Research, its New Zealand equivalent, has been. Allen says he hopes that CSIRO is now "secure" against being split up, and describes that part of the CSIRO's work which is for the public good as not being "directly saleable". □

Axe the army of cheap labour

SIR — The implication of a recent News story, "Cash rivalry 'fosters neglect of postdoc supervision'" (*Nature* **383**, 287; 1996), that injecting more money into the National Institutes of Health or the National Science Foundation or other US funding agencies will solve the current problems of doing science, is patently wrong. There is a much deeper problem here — there are too many scientists.

The university professor is under tremendous pressure to publish a lot of papers to support his or her next grant application/renewal. Laboratories are filled with cheap labour in the form of graduate students and postdocs (particularly foreign postdocs). Unless an army of this cheap labour is in place constantly, the professor cannot publish the necessary number of papers to show apparent productivity to get funding, thus creating a vicious circle. Meanwhile, graduate schools are flooded with ever-increasing numbers of students because the professors are scrambling to feed the workers in to the vicious circle of grant-getting.

The only way to break the cycle is to reduce the number of graduate student admissions and to cut down the influx of foreign postdoc scientists. Right now the system is overloaded. The average science job opening gets hundreds of applications. In this madding crowd of scientists, nice words such as 'mentoring', 'collegiality', 'supervision' and 'teaching' are lost. The belief that Darwinian and 'market forces' will somehow correct the current problems is a fantasy. Meanwhile, highly educated hard-working scientists are underpaid and undervalued by everybody, including fellow scientists.

Srin Sastry

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SIR — The relentless decline of scientific culture, the venality of senior researchers, the torments of junior academic scientists battle-scarred by the blunderbuss of the National Institutes of Health and the National Science Foundation — these things always make news. But is it really worthwhile to devote two-thirds of a precious News page (*Nature* **383**, 287; 1996) to the lurid — and to my mind wholly meretricious — theme of modern molecular biology postdocs oppressed by their mentors, the ageing scientists terrified by the prospect of competition from their former co-workers?

I applaud Julian Jack's initiative through the Burroughs-Wellcome Trust to award to outstanding postdoctoral researchers innovative grants that continue into their post-

postdoctoral, independent research years. But I retch at the notion that this most welcome programme is motivated, as Jack claims, by a "drop-off in mentoring" because postdoctoral supervisors, these days, crudely perceive their young charges as future competitors for grant money.

There are, of course, egregious instances of emotionally insecure senior scientists thwarting their newly hatched postdocs by forbidding them to work in competitive areas. But Jack's belief that in the face of the funding crunch this odious practice is now widespread strikes me as objectively unsupported. On the contrary, most postdoctoral advisers adhere to a pervasive cultural tradition, viewing their mentoring function as a two-pronged responsibility.

First, they try to point their trainees in productive and practicable research directions. When this works, it's of great advantage to both mentor and mentored. (I frequently remind my postdocs that their only reason for existence is to make me famous.) Second, most advisers are aware that they must provide opportunities for the emergent researcher to elaborate a good project on which to make a first independent 'hit' and establish, as early as possible, a personal reputation for scientific excellence. Sometimes this means handing a pet project over to a promising beginner going into a first job and standing discreetly apart from it for a while. This is not news. Just about everyone does this without fuss or fanfare. Senior scientists who fail to do it are viewed by their peers as fundamentally deficient.

Like parents watching their children enter the adult world, most senior researchers consider that being surpassed, one-upped, outfoxed, or scooped by a former postdoc is a great joy, a talisman of success. It is this tradition that propels the fast-moving front of discovery, which by its very nature must be quickly grasped, along with the culture subsuming it, by succeeding generations.

Christopher Miller

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SIR — A PhD degree, by most university standards, trains a scientist to be capable of independent research. So it is sad to read of postdoctoral scientists routinely referred to as 'students'. The freedom of postdoctoral scientists has been systematically eroded over the years and now we are looking for an excuse to justify that loss.

If it is to have any meaning, 'postdoctoral training' should mean that the postdoc has the opportunity to develop skills that

cannot be learned during a PhD — supervision of students or staff, developing and carrying through independent research (to the point of publication) and developing skills at collaboration.

Yet postdocs are rarely allowed the freedom to develop such skills, ironically on the basis that they are not fully fledged scientists, but students. Thus the concept of postdoctoral training actually hinders postdoctoral development.

Senior scientists conveniently forget that James Watson and Francis Crick were postdoc and student respectively when they did their Nobel prizewinning work. If the attitudes of today had existed then, would they have had the freedom to do that work or been permitted to publish without their respective group leaders? In both cases I believe the answer is 'no'. Some time between then and now, some great ideal of science, the ideal of unfettered research at all levels, has died, so gradually that no one mourned its passing.

Gerard Vassiliou

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Back to the roots

SIR — It is unfortunate that the British publisher to whom Koreaki Ito submitted his paper (*Nature* **382**, 666; 1996) still uses the spelling 'disulphide' rather than 'disulfide'. The Royal Society of Chemistry in the United Kingdom has been using the spelling 'sulfur' since 1992, and at the time of the change mentioned the desire to come into line with other English-language usage.

The name of the element is thought to have come originally from the Sanskrit word 'sulvere'. British lexicographers are thought to have taken the name of the element to have a Greek root, hence the -ph-, whereas their US counterparts took the name to have a Latin root, hence the -f-. (Interestingly, in Lassen Park in California there is a conurbation called Sulphur Works.) In the interests of homogeneity and ease of abstraction, the spelling 'sulfur' should be used by all English-language publications.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only, or e-mailed to corres@nature.com

Collaboration still the key to Rosetta

SIR — Your News article about the decision of the US National Aeronautics and Space Administration (NASA) to reduce its commitment to the Rosetta mission (*Nature* **383**, 469; 1996) merits praise for bringing to general attention the unfavourable evolution of international collaboration in space science.

It is regrettable that, when budgets decline and opportunities for national scientists decrease, the will to find a place for international partners decreases. We should fight this trend, and the European Space Agency (ESA)'s Science Programme is committed to offering as many opportunities as possible for international cooperation.

From this perspective, I should like to express some reservations about your article, whose stress on the negative aspects can only aggravate the situation.

As the article correctly states, both agencies regret what has happened. And, although it is true that "the United States has pulled out as a major contributor" to the Rosetta mission, it has not pulled out altogether. At the meeting in Washington in September, NASA stated its intention to: continue support to the three US principal investigators on the Orbiter, and consider

support to the US co-investigators; continue support to the previously selected US interdisciplinary scientists; provide a reasonable level of support through the US deep space network; assist, when requested by ESA, in assessing the technical status and progress of any aspects of the Rosetta mission (including the lander); and consider funding any US experiment that could be included in the remaining Rosetta lander.

International collaboration is becoming difficult; let us not make it more difficult by creating reasons for bitterness where there should be none.

R. M. Bonnet

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Female forum

SIR — I was outraged and saddened by Dan Graur's letter (*Nature* **383**, 116; 1996) about the conference on "Women in Evolution" held at the University of Arkansas in September.

As a participant and workshop discussion facilitator at this conference, and as the bearer of a single X chromosome, I should like to respond. It is true, as Graur points out, that only women were invited as guest speakers, but the conference was not limited

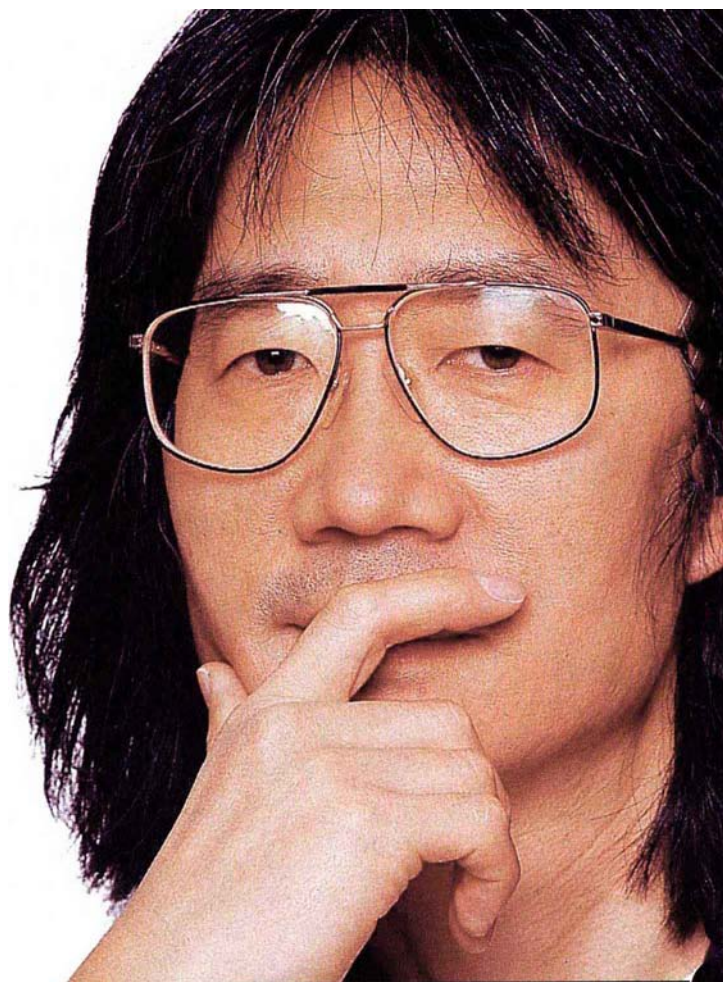
to women. Nearly a quarter of the registrants were male (30 of 134). Graur was not excluded.

Graur's cynical letter leaves the impression that women have not been discriminated against in the field of evolutionary biology. Most informed individuals agree that women have indeed been marginalized and excluded in the past. Although there is less difference in academic rank between men and women in biology than in other scientific fields (*American Scientist* **84**, 63-71; 1996), there is poor representation of women in biology faculties in the United States and elsewhere. When academic couples near the completion of their respective PhDs, women are more likely to forgo their careers in deference to their male partners because of the perception that men advance in their careers more readily than women.

As the organizer, Sydney Cameron of the University of Arkansas, said, the conference goal was "to present some of the key areas of evolutionary biology" and "to discuss important issues concerning women and science". By featuring women as speakers, the conference demonstrated the achievements of women in evolutionary biology and the social and professional climate within which they have worked.

Women and men are not treated equally in academic science, including evolutionary biology. Conferences that feature women speakers can help to convince male partici-

To **save an hour**
each time you
purify histidine-
tagged proteins,
put it on the
tip of a syringe



pants of the fundamental equality of the sexes in academic life and to provide women with the self-confidence and positive role models necessary to achieve this equality. That is, after all, the point of women's colleges, which continue to contribute disproportionately to leadership ranks in the United States.

As a future male biology faculty member, who feels privileged to work in a department in which 43% of the faculty are women, I found the conference important in illustrating some of the problems that women face and how those problems might be rectified in the future. It was clear to the sponsors that a conference on "Women in Evolution" would be productive and special, and Graur seems to have missed the point that most conferences are planned by and for "partially bald, middle-aged, pot-bellied individuals".

Seth Isenberg

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Back to nature

SIR — I was puzzled by the suggestion in your leading article "Distrust in genetically altered foods" (*Nature* 383, 559; 1996) that the genetic tailoring of crops is "precisely

the opposite direction" from "increasing demands for 'natural' products". Turning to a familiar paragraph of Rachel Carson's *Silent Spring* (1962), I read:

"A truly extraordinary variety of alternatives to the chemical control of insects is available. Some are already in use and have achieved brilliant success. Others are in the stage of laboratory testing. Still others are little more than ideas in the minds of imaginative scientists, waiting for the opportunity to put them to the test. All have this in common: they are biological solutions, based on understanding of the living organisms they seek to control, and of the whole fabric of life to which they belong. Specialists representing various areas of the vast field are contributing — entomologists, pathologists, geneticists, physiologists, biochemists, ecologists — all pouring their knowledge and their creative inspirations into the formation of a new science of biotic controls."

That seems a fair and farsighted summary of the development of biotechnology in the following three-and-a-half decades, in one of its main fields of application. Subtlety and precision are widespread in nature; it is sad, therefore, to see their use by man stigmatized by *Nature* as unnatural. Molecular biology is part of the "back to nature" movement.

Mark Cantley

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Making waves

SIR — A. G. Gordon's proposed explanation of the function of the stapedial muscle as a noise protection system triggered by the tactile pressure produced by a blast wave impinging upon the outer entrance of the ear canal is certainly interesting, but unfortunately rather nonsensical from a physical standpoint (*Nature* 382, 665; 1996).

When a pressure wave in a gas becomes sufficiently strong, it steepens into the classic N-shaped wave profile (if one assumes an initially sinusoidal waveform) of a blast, or shock, wave. Shock waves differ from simple sound waves not only in that there is a much larger pressure rise in the fluid associated with their passage, but also in that they travel supersonically. Thus, while a quickly travelling blast wave would indeed arrive at the ear of the listener first, he need not be concerned about the noisy acoustic waves following behind it, as the most dangerous 'noise' from the explosion is the blast wave itself, which would shatter his eardrums (and possibly remove most of his clothing) well in advance of any loud, but conventional, acoustic sounds.

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Stop knocking social sciences

SIR — 'Scientific misconduct' has been much discussed recently, leading at least one commentator to suggest that "the current research environment seems to foster cynicism about simple virtues such as honesty and fairness"¹. Consider a case in point.

I edit a journal, *Social Studies of Science*, which serves a growing international community of researchers, mainly in the social sciences and humanities, working on a wide range of problems and issues. Many members of this community collaborate closely with scientists and engineers, earning their credibility through effective work in industry and public policy. These individuals have recently come under attack from a number of scientists, mainly in the United States. Authors find their work incompetently summarized and unfairly derided in what one reviewer has described as a "willful strategy of distortion and demonization"². Some have been personally insulted, and many are being actively harassed, particularly on US campuses.

In publicity material for a recent conference organized by the New York Academy of Sciences, it is implied that the community's work consists of "arguments advanced by those who would reject reason and take up the cudgels against science". Critics have dismissed their research as "a body of work founded on silly philosophy, sloppy history, anemic research, boundless ignorance, and just plain lousy scholarship", exhibiting "a deeply disapproving stance towards science, sometimes combined with a profound ignorance of it"³. Ironically, such intemperate claims are being made in defence of scholarly standards and values.

I cite just two specific examples. Steven Weinberg⁴ misrepresents the work of Edinburgh colleagues (without evidence or citation), with two offensive 'jokes' — one describing the so-called "strong programme" in the field as the "Strong Pogrom", the other referring to "madness" and "nonsense". Elsewhere⁵, a book by two reputable sociologists of science has been dismissed as "largely claptrap, but... attractive to... students looking for an easy path to a degree... just another product of academics on the make... who interpret carefully selected data to fit their prejudices, all to improve their standing in the academy without having to do any intellectual heavy lifting".

Such unscholarly language is unacceptable in intellectual debate. One could, perhaps, brush aside this kind of thing, much as we British feel able to ignore (say) the creationists. However, where individuals expressing such views wield power in the academic world, the possibility of corrupting the scholarly process is real. It should be resisted firmly. Scientists may feel that perceived threats to their 'objectivity'

justify crude responses, and abandon all traces of trust, sensitivity and respect among academic colleagues. But such a strategy is counterproductive: far from conserving and extending the 'science and reason' it claims to be defending, it actually demeans and devalues it.

This behaviour is clearly a breach of any guidelines for proper conduct among scientists. One immediate casualty of this tawdry campaign could be the academic credit and insight so painstakingly acquired by our community over some 30 years, and now available to inform and guide progress towards the fuller 'science and reason' demanded by our increasingly complex society. To squander so carelessly what we have learned so patiently would be tragic.

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Abstract values

SIR — The recent survey by Liu and Danziger¹ was not the first on the fate of conference abstracts. Goldman and Loscalzo² followed up 276 randomly selected cardiology abstracts, and about half became full-length articles in peer-reviewed journals after 3.5 years. Meranze *et al.*³ looked at anaesthesia abstracts 1–2 years after presentation, and Yentis *et al.*⁴ did a more extended survey of abstracts from four anaesthesia societies. Again, about half had been published by 3 to 5 years. Scherer *et al.*⁵ surveyed abstracts in ophthalmology; about half appeared in full, mostly within 2 years, and there was a weak association between full publication and statistically significant results. Liu and Danziger's figures for their four journals were lower than 50%, though some of their 1992 abstracts are not yet 5 years old.

Articles on this subject can be found in Medline by searching on the word 'abstract' as a title word, and then using the terms 'abstracting and indexing', 'congresses' and 'time factors'.

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No miracle

SIR — Your reports on the production of 'herbal petrol' by Ramar Pillai of Tamil Nadu in India may have given the impression that his claim has been substantiated (*Nature* **383**, 112; 1996). Not only is that not true, but it is clear that he is a hoaxer, and that the petrol he has been producing in his demonstrations did not come from a plant.

Pillai has been making his claim for some time but, whenever experiments have been carried out under controlled conditions, they have been total failures. The first such experiment was in early 1995 at the Centre for Policy Studies in Madras, when Pillai apparently abandoned his equipment and disappeared. The second was carried out a few weeks ago under the supervision of Professor V. S. Ramamurthy, secretary of the Department of Science and Technology of the Government of India, and was again a disaster for Pillai, with not a drop of petrol forthcoming; he was caught trying to introduce commercial petrol into the system on the sly (see *Nature* **383**, 269; 1996).

A large number of reports have since appeared in Indian newspapers expressing doubts about Pillai's claims, as well as providing a reason for his deceit. There have been large-scale thefts of petrol from tankers in and around the area in which Pillai lives, and the strategy seems to have been to sell this petrol on the open market as 'herbal petrol'. It is widely believed that individuals with political links have been involved, knowing that they could exploit the gullibility of the many people, including scientists, prepared to believe in miracles.

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Spanish practice

SIR — J. T. C. Sellick (*Nature* **383**, 569; 1996) asks how to keep two surnames when authoring a paper. He complains about all sorts of combinations made by indexing databases which, being accustomed to handling only one-surnamed authors, find it difficult to consider other possibilities.

In Spain we have two surnames inherited from each of our parents and we all have similar problems when deciding what will be the 'scientific name' that will be used to author a paper. Some people, like myself, settle on only one surname, usually the less common one, but those who wish to keep both surnames resort to using a hyphen between them. This option seems to keep journal editors and database curators happy. I therefore suggest to J. T. Clark Sellick that he changes his name to J. T. Clark-Sellick.

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On the likelihood of habitable worlds

John Maynard Smith and Eörs Szathmáry

The anthropic principle has gained much popularity among cosmologists. But, faced with a need for historical explanation, biologists are bound to find it a cop-out.

THE anthropic principle suggests that physical laws can be explained by the fact that they have given rise to intelligent observers, able to discuss their nature. The idea has gained considerable popularity among cosmologists, but biologists are bound to have reservations. If it is true that "we are here because we are here", the study of evolutionary transitions becomes the study of the trivial or the futile. We suggest that the main merit of the anthropic principle may be that it has aided the appearance of other, potentially more useful, approaches to the problem of the likelihood of habitable worlds.

The idea that the world is peculiarly adapted to the appearance of life is not a new one. In 1913, the biochemist L. J. Henderson¹ pointed out that many substances such as water have precisely those properties required if life is to exist. Most biologists rejected his views, arguing that organisms are adapted to their environments by natural selection, not the other way around. But the questions he raised have surfaced again recently in a new form. It turns out that the physical constants have just the values required to ensure that the Universe contains stars with planets capable of supporting intelligent life. The 'cosmological anthropic principle'² has been suggested as an explanation for this puzzling fact.

The principle takes several forms. The weak anthropic principle merely states that certain universes, with unfortunate lists of physical constants, would not be observable by us, simply because we would not be there. The weak principle is not a theory: it merely acknowledges a peculiar situation.

The strong principle, proposed by Brandon Carter³, is more radical. It states that the Universe *must* have those properties that allow life to develop in it at some stage of its life history. How can this curious claim be understood? The simplest interpretation is that the Universe was designed by a creator who intended that intelligent life should evolve. This interpretation lies outside science. Within science, there are two possibilities. First, there is only one universe possible on logical grounds, and the list of constants follows from a (so far unavailable) 'theory of everything'. Second, there are indeed many possible alternative universes. If so, the presence of observers may have a crucial role, since, according to the Copenhagen interpretation of quantum physics, it is the act of observation that chooses among possible

superpositions. This version depends on the perhaps unjustified assumption that Schrödinger's equation can be applied to macroscopic objects. It also seems to lead to the rather odd conclusion that the wave function did not collapse until the recent evolution of conscious observers on Earth, or perhaps elsewhere in the Universe.

We acknowledge the value of the weak anthropic principle in putting a constraint on cosmological theories: some models are incompatible with our very existence. But this is not the same as an 'explanation' of physical laws, at least as the word is commonly used. To explain an event is to give a cause for that event. This is the sense in which the word is used in the biological sciences, although perhaps not in physics. Of course, an event may have several causes. That the heart beats requires both a physiological explanation, in terms of the properties of muscles and nerves, and an evolutionary explanation, in terms of natural selection for efficient transport. The strong anthropic principle seems to be an explanation in this causal sense, asserting that intelligent beings *must* come into existence. The assertion is essentially unproved, and unlikely to be true. If we had to come about anyway, then analysis of evolution, seen from this high level of 'explanation', is almost irrelevant.

Evolutionary biology is a historical science. It tries to explain past events in terms of a theory (natural selection — that is, the dynamics of populations of entities with variation, multiplication and heredity). To explain a particular event, say the origin of the eukaryotes, is to show that, given plausible initial conditions, the event, if not inevitable, was at least reasonably likely. The explanation should also be supported by evidence: the symbiotic theory of the origin of the eukaryotes is supported by the presence in mitochondria of DNA and a bacteria-like translating machinery. It would be unsatisfactory to argue that, because eukaryotes are in fact here, then any accidents, however unlikely, needed to give rise to them must have happened.

As biologists, we are unhappy with the anthropic principle because, faced with a need for historical explanation, it seems to be a cop-out. We are correspondingly attracted by Smolin's recent suggestion⁴ that the values of the physical constants can be explained by a process of cosmological natural selection. The central idea is that the

formation of a black hole is equivalent to the formation of a new 'universe', causally isolated from the 'parent' universe. The word 'universe' is here being used to mean not 'everything there is' but a causally isolated system. If, as J. A. Wheeler has suggested, the laws of nature in the new universe differ by a small amount from those in the parent, we have the properties of variation, multiplication and heredity needed for natural selection. The 'fitness' of a universe — the property maximized by selection — is the number of black holes produced. Smolin predicts that any small change in the constants should reduce (or leave unchanged) this number. The physical constants needed to maximize the production of black holes correspond, roughly, to those needed for the appearance of stars, planets and perhaps observers. So the theory offers a causal explanation for the fact that the constants are peculiarly adapted for the appearance of intelligent life.

We can see some difficulties. Most seriously, models of natural selection in biology always assume that the total population size is limited (by space, nutrients or what have you), an assumption true of real populations, except for short periods of time. A population of causally isolated universes would not be limited. In Smolin's model, even inferior universes, with a smaller productivity of black holes, multiply exponentially, even though they constitute an ever-decreasing proportion of the whole population. So given two types of universe, with different productivities of black holes and hence different Malthusian fitnesses, both would multiply exponentially, and reach infinite numbers in infinite time, but the proportion of the fitter type would approach unity. If the physical constants needed to produce black holes also favour the appearance of life, then the probability that a random universe will be favourable for life will also increase. □

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A new opportunity for science in Europe

Françoise Praderie*

Closer integration between European research and the needs of society would have a positive effect on the development of Europe while helping to shape the future of its scientific communities. To promote those aims we intend to create 'Euroscience', an open association of all those interested in science and its consequences on society.

THE relationship between science and the public can be comfortable (as in the public's continuing fascination with astronomical discoveries) or stressed (see, for example, debates concerning the implications of genetic analysis and modification). The links between rapidly expanding human knowledge and the evolving needs of society are never static, and merit continuous appraisal, as do the activities of the various groups of participants. In turn, such appraisal should lead to feedback that can stimulate and enhance those to whom it is directed.

We believe that, at a European level and within many European countries, there is inadequate opportunity for such feedback. But we also believe that new opportunities can be created. As a result of enhanced consideration and dialogue, new partnerships between society and science can be invented: scientific approaches to society's demands can be more effectively and considerably matched; the partnerships can better adapt to changes in the process of knowledge generation and also respect the intrinsic constraints and uncertainties of basic research; and they can take account of changing conditions in the public perceptions of science. Another anticipated benefit is that scientists can become more aware of society's needs and concerns, and be more ready to invest their expertise in tackling them.

In addition, there is a need for a higher degree of integration between the scientific communities of European countries. Despite efforts to promote a better co-ordination of European research through common structures (especially for large facilities), national research programmes are still insufficiently interactive, hindering the ability to reconcile expanding knowledge and the interests of those it serves.

We have begun the process of establishing an organization intended to address these problems. Called Euroscience, it has already attracted members from Europe's research community as a result of personal contacts. The purpose of this first public announcement is to invite others to become involved.

In the most general terms, Euroscience is intended to analyse perspectives of science and the future of the scientific

workforce in Europe in the light of challenges faced by European countries and their societies. There is a clear analogy with the role in the United States of the American Association for the Advancement of Science.

More specifically, the initial priorities of Euroscience will be to:

- investigate more effective strategies to define and address society's demands in terms of science and technology;
- define ethical guidelines for evaluating and managing science programmes and disseminating their results;
- appraise prospects for the careers and employment of young scientists and the adequacy of current education systems (for instance, postdoctoral training and guidance), in the context of society's changing needs;
- promote closer integration of the scientific workforce throughout Europe at large and thereby preserve a necessary diversity of available skills in research;
- learning from the experience accumulated in some countries, take initiatives that would improve the understanding between scientists and the public of the roles of science and of scientists in our societies;
- address Europe's responsibilities towards other countries developing through science and technology;
- act as an additional channel for the views of scientific communities towards government bodies, including the institutions of the European Union, as well as national agencies and research councils.

To these ends, Euroscience will provide open forums for scientists, teachers and students in all fields of humanities, social and natural sciences, for those interested in developing science and technology, and for anyone interested in these issues. Forums should result in practical proposals to implement Euroscience's objectives, for instance by addressing reports to science agencies, government bodies, parliaments, the media and others.

Complementarity

Euroscience is not intended to be a learned society, an academy or a trade union, although it plans to work in cooperation with all institutions sharing similar goals, such as European learned societies, the European Science Foundation and Academia Europaea. It is open to anyone having

a vested interest in science, technology and their impact on society: teachers; scientists actively involved in research and education (academic and industrial, young and established, representing all areas of science and engineering including social sciences and the humanities); and partners of science from the media, industry, science management and consumer groups.

Initial activities

A document "Call to European scientists for the creation of a large, multidisciplinary movement for the promotion of science and technology in Europe" was drafted in April 1996 and circulated by a small group of initiators. It received immediate support from 145 distinguished scientists from 22 countries and all disciplines, hereafter referred to as "founding members". They agreed to help launch Euroscience. They represent a fair balance of disciplines, and are drawn from across the whole of Europe. A list of founding members is available from the e-mail address in the box below.

Most founding members were selected because of their high scientific visibility and broad political views of science, to achieve scientific credibility at this initial stage. That explains why the first call for founding membership was relatively restricted. However, all founding members insist that Euroscience should be open to anyone sharing our concerns.

A meeting of founding and potential members will convene on 15 March 1997 in Strasbourg. It will elect a provisional Board, including a chairperson and vice-presidents, responsible for proposing by-laws, preparing elections and initiating priorities to be discussed on that occasion. Financial resources will be sought on a regular basis through individual fees, and through gifts and donations from institutions in the launch phase. □

Contacting Euroscience

Europeans from all countries of geographical Europe who share these views and would like to join the Euroscience project can obtain additional information by writing to:
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*On behalf of the founding members of Euroscience.

Pictures of MHC restriction

Peter Parham

This year's Nobel prize in physiology or medicine was awarded for the discovery, in 1974, of a fundamental principle in immunology. The underlying interactions are now being seen in three dimensions.

CYTOLYTIC T lymphocytes are white blood cells that prevent the spread of viral infections by killing infected cells and putting a stop to virus production. Almost a quarter of a century ago, Rolf Zinkernagel and Peter Doherty serendipitously found that these lymphocytes sense infection of a cell by identifying viral antigens displayed at the cell's surface in combination with major histocompatibility complex (MHC) class I glycoproteins¹. Discovery of this phenomenon, which is known as MHC restriction and won Zinkernagel and Doherty a Nobel prize this year, stimulated a grand movement to identify the molecules involved in the game and the rules by which they play.

The way forward has been marked regularly with milestones, many of them stemming from structural biology. The latest, the three-dimensional structure of the T-cell receptor (TCR) and its interaction with MHC class I, are reported by Garcia *et al.* in the 11 October issue of *Science*² and by Garboczi *et al.* on page 134 of this issue of *Nature*³.

Restriction of viral antigen by a class I MHC molecule stems from direct interaction between the two — it is not the native proteins or glycoproteins of a virus that bind to class I, but small peptide fragments produced by degradation of the virus inside the cell⁴. The intimate nature of the contact was first seen in the crystal structure of the human class I molecule, HLA-A2, where electron density due to a mixture of peptides was embedded in the class I molecule⁵. Additional structures of human or mouse class I loaded with homogeneous peptides revealed the extended conformation of the bound peptide, with its side-chain anchors filling complementary pockets of the MHC binding groove^{6,7}.

T-cell receptors are built on the same principles as antibodies: they have α and β polypeptide chains, which are the products of rearranging genes and consist of variable and constant regions⁸ (Fig. 1). From comparison of amino-acid sequences, the four extracellular domains of the TCR were predicted to resemble the antigen-binding fragment (Fab) of immunoglobulin. It was also proposed that TCRs have variable-domain loops that correspond to the complementarity-determining regions (CDRs) of antibodies and which would bind to MHC and peptide⁸⁻¹⁰.

Unlike antibodies, TCRs are in short

supply in nature. Structural studies can be voracious in their consumption of protein, and genetic engineering was the only possible means to satisfy the demand for material. The quest for suitable preparations has been a chaotic exercise in trial and error, for which the hopes, fears, promises and disappointments are readily tracked

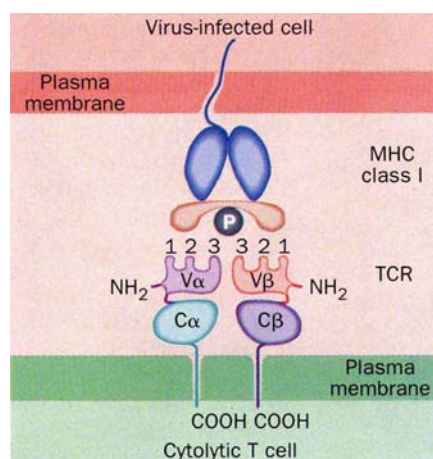


FIG. 1 The T-cell receptor (TCR), and its interaction with a class I major histocompatibility complex (MHC) glycoprotein plus viral peptide (P) displayed at the surface of a virus-infected cell. The extracellular part of the TCR consists of α - and β -chains, which have variable (V) and constant (C) domains. 1, 2 and 3 are the three complementarity-determining regions (CDRs), which are the parts of the TCR that actually interact with the MHC molecule and peptide. This diagram does not depict the precise contacts between MHC and TCR, which are shown in simplified form in Fig. 2.

in the literature and the *Wall Street Journal*. Finally, Mariuzza and co-workers solved structures for the TCR α - and β -chain separately and proved that they are made from immunoglobulin-like variable and constant domains. Their ground-breaking papers^{11,12} set the stage for the structures of the TCR heterodimer and its complex with MHC plus a viral peptide^{2,3}. (Coincidentally, Mariuzza and co-workers describe another structure, a complex of TCR β -chain and superantigen, in a paper that also appears in this issue¹³.)

Since Zinkernagel and Doherty's famously sparing letter to this journal¹, T-cell science has moved with the times. In 11 fact-crammed pages, Garcia *et al.* supply a manual for the mouse TCR that verges upon the encyclopaedic. They find

the earlier predictions to be largely correct: "the α and β chains [of the TCR] fold into a quaternary structure that resembles the antigen binding region (Fab) of antibodies", in which the "variable domains resemble antibody variable domains". These and other points made by Garcia *et al.* are testament to the expanding knowledge of protein sequence and structure and its potential for prediction — at least when combination of known elements produces novel variation on a familiar theme. An unexpected bonus in the TCR structure is the presence of ordered carbohydrate, which proves, without resort to biochemical manipulation, that four of the seven potential sites of glycosylation are indeed glycosylated. Such sugars may well have facilitated crystallization of the notoriously difficult heterodimer. Without doubt, structure mavens will luxuriate in the body of information provided by Garcia *et al.*, but the suspense might prove too much for the general reader, for it is only at the third column of the ninth page that the immunological revelation begins.

Although their paper dwells on TCR alone, Garcia *et al.* also have crystals of TCR bound to MHC class I and viral peptide. Description of this structure appears to be at a preliminary stage, but by imposing the known and separate structures of class I and TCR upon the data they have, Garcia *et al.* manage to peep in the face of MHC restriction. This theme is more forcibly taken up by Garboczi *et al.*³, who have brought to higher resolution their analysis of co-crystals of HLA-A2, a peptide derived from the human T lymphotropic virus (HTLV-1), and human TCR.

In terms of the basic topology of the interaction, the two papers reach similar conclusions. Interrogation of MHC-peptide complexes by TCR (the basis for T-cell selection and specificity) occurs between opposing, rectangular faces of a similar size. The two faces are neither congruent nor orthogonal, as has been previously speculated, but diagonally orientated (Fig. 2), with the set of CDR loops aligning with the β -strands that form the base of the class I binding groove. This arrangement facilitates an interlocking of the TCR and MHC structures, one which Garboczi *et al.* assert is the essence of all interactions between TCR and MHC.

In binding to MHC, the TCR α - and β -chains appear to brace the peptide-binding

groove. The TCR α spans the left end of the class I binding groove, with its first CDR loop covering the amino-terminal part of the α_1 helix and the second CDR covering the carboxy-terminal part of the α_2 helix. In complementary fashion TCR β spans the right end of the groove, with its first CDR loop covering the carboxy-terminal part of the α_1 helix and the second CDR covering the amino-terminal part of the α_2 helix. Through these contacts, TCR α covers the amino-terminal half of the peptide while TCR β covers the car-

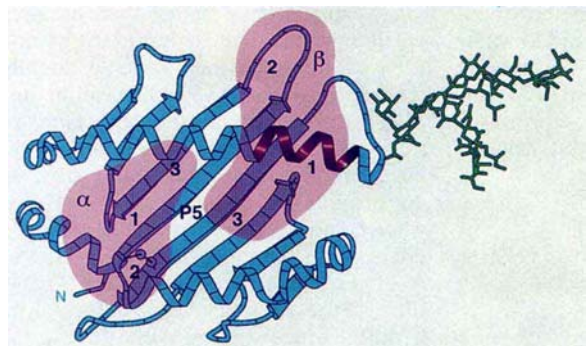


FIG. 2 Interaction of the T-cell receptor with the class I MHC molecule. The blue ribbon diagram of the α_1 and α_2 domains of the HLA-A2 molecule is based on that of Bjorkman *et al.*⁵. The regions of interaction of the TCR α - and β -chains are shown in pink, with the rough positions of the CDR loops shown by the numerals. P5 denotes the hydrophobic pocket which is formed by the third CDRs of the α - and β -chains and which contains the side chain of tyrosine 5 of the HTLV-1 peptide. The oligosaccharide covalently attached to asparagine 86 of the HLA-A2 α_1 domain is shown in green and is based on the model of Barber *et al.*¹⁶. The orientation of the carbohydrate with regard to the protein domains is not known and it is shown 'pointing away' for graphic convenience. The carboxy-terminal region of the α_1 helix of the class I molecule that interacts with the inhibitory receptors of human natural killer cells and T cells (KIR) is shown in purple.

boxy-terminal half. Garboczi *et al.* describe the burial of almost the entire viral peptide in the TCR/MHC interface—a brave image of the human immune system interring its broken and degraded enemy.

The two ends of the HTLV-1 peptide are tightly anchored into pockets of the HLA-A2 binding groove, whereas the central region arches up out of the groove to confront the third CDRs of the TCR. The tips of the third CDRs of the α - and β -chains are separated by a deep hydrophobic pocket which binds the side chain of the fifth of the nine amino acids in the HTLV-1 peptide. Although most peptides that bind MHC class I comprise nine amino acids, minority populations are of different length. For example, Garcia and colleagues' peptide has eight amino acids, and it is the side chain of the fourth residue that fills the pocket made by the third CDRs.

The importance of the third CDRs in determining antigen specificity was predicted on the basis of amino-acid-sequence comparisons^{8–10} and shown from the results of immunological experiments that probed TCR–MHC interactions¹⁴.

The contributions of the first and second CDRs, and the relative orientation of MHC and TCR, have been more contentious. It is here that the new structures will have their greatest impact.

Garboczi and colleagues' data are at sufficient resolution for them to identify the contacts between MHC, peptide and TCR. For both the α - and β -chain, the first and third CDRs contact peptide. However, whereas all three CDRs of the α -chain bind the MHC helices, only the third CDR of the β -chain has this function. It is striking that the second CDR of the β -chain "although positioned over the α_1 domain [helix] does not make any contacts". As a consequence, TCR interactions are more extensive with the α_2 helix of class I than with the α_1 helix, a distinction anticipated by the relative concentration of peptide-contacting residues in the α_1 helix of HLA-A2 and of TCR-contacting residues in the α_2 helix^{5,15}. Despite the correlation, it remains intriguing that the TCR hangs over the carboxy-terminal half (residues 73–84) of the α_1 helix but makes no contact.

One explanation is that the MHC class I molecules used by Garboczi *et al.* were made by bacteria and lack the invariant oligosaccharide normally attached to residue 86 near the carboxy-terminal end of the α_1 helix (Fig. 2)¹⁶. If the carbohydrate were present it might change the

picture, either by creating interactions itself or by modifying the protein–protein contacts. Another possibility is that TCR avoidance of residues 73–84 is related to the fact that a second set of MHC class I receptors, those found on natural killer cells and certain T cells, bind to residues 74–83 of the α_1 helix¹⁷. Thus these killer-cell receptors (called KIR for killer-cell inhibitory receptors, though some appear to be stimulatory) bind to the region of the α_1 helix that TCR does not. There is the tantalizing possibility that TCR and KIR sometimes bind simultaneously to the same class I molecule, thereby acting in concert or competition to alert or sedate a T lymphocyte.

The latest paper from Mariuzza and co-workers (Fields *et al.*, page 188 of this issue¹³) describes the structure of staphylococcal enterotoxin bound to the TCR β -chain. The principal contact site of the β -chain is none other than the second CDR, the one CDR that does not contact MHC in the structure of Garboczi *et al.* By combining this new structure with those described previously for the toxin bound to

MHC class II (ref. 18) and for the TCR α -chain¹², Fields *et al.* model how toxin can enhance the binding of TCR to MHC class II containing nonspecific peptides (MHC class II is structurally very similar to MHC class I, but presents peptide antigens to inflammatory and helper T cells). The authors predict that the TCR α -chain binds to the MHC class II molecule (to the part equivalent to the α_2 helix of the class I molecule; Fig. 2). In contrast, the TCR β -chain and MHC class II have no direct contact but are bonded by the toxin, which glues the TCR and MHC so they no longer need the finicky interaction between peptide and TCR. In this way, the bacterial superantigen sidesteps the normal requirements of T-cell activation and can trigger the huge populations of T cells that cause food poisoning or toxic shock. In Fields and colleagues' dark image, the human immune system is sabotaged by the enemy's invention.

Following Zinkernagel and Doherty's discovery of MHC restriction of viral antigens, there was debate as to whether one or two T-cell receptors were involved. The first model proposed that a single receptor contacted both MHC and viral antigen, whereas the second envisaged two separate receptors; eventually the weight of evidence came down on the side of the single receptor, as was persuasively reviewed by Matzinger¹⁹ in 1981.

When polled about the award of the Nobel prize to Zinkernagel and Doherty, Matzinger implied that it was long overdue²⁰. For the cellular immunologist that could well be true, but for those who dream in molecules the timing seems right. To such as them, the opening by Garcia *et al.* and Garboczi *et al.* of an exhibition of pictures of MHC restriction is ultimate proof of the inspiration that Zinkernagel and Doherty¹ wrested from virus-infected mice and the cellular assays of T-cell cytotoxicity. □

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A nanotube molecular tool

David Keller

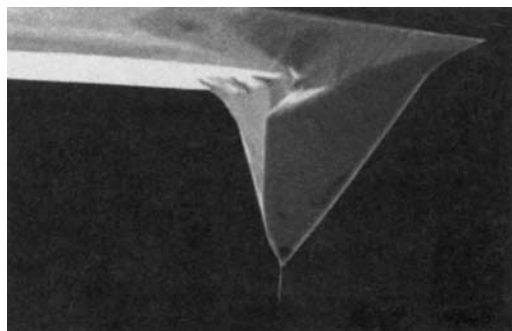
SINCE their invention in the early 1980s, scanning probe microscopes have become the tool of choice for probing and manipulating the nanoscale world. Smalley and colleagues (Dai *et al.*, page 147 of this issue¹) have now given the brush a finer point by securing a single carbon nanotube to the tip of a scanning force microscope (SFM). The nanotube tip can gain access to deeper recesses of surface structure than can the very much blunter pyramidal tip of a standard device.

With the advent of the scanning tunnelling microscope (STM), it became possible (in principle) to build structures on the atomic scale in the same way that we do on the macroscopic scale: by putting together individual components (atoms or molecules) one at a time. The dramatic experiments of Eigler and others, who used an STM to assemble tiny rings, letters and other complex structures from individual adsorbed atoms on metal surfaces², showed that even single atoms can be visualized and manipulated. Many other experiments have demonstrated a wide range of measuring, imaging and nanofabrication capabilities on somewhat larger scales^{3,4}.

The STM (or the related SFM) is uniquely suited to these kinds of 'single molecule' experiments because it combines the ability to visualize individual atoms and molecules with the ability to manipulate them. Both feats are accomplished by bringing an atomically sharp probe tip very close to the sample surface and measuring some sort of tip-sample interaction (the actual quantity measured depends on the type of experiment). Then, by scanning the probe over the surface, a high-resolution image can be created; or, by changing the tip-sample interaction (say, by bringing the probe tip closer or by applying a voltage pulse), the tip can be used as a tiny tool to alter the same sample it previously imaged. A second image shows the results of the operation.

Clearly, the probe tip is the crucial element in any such experiment. Both the resolution of the STM when used as a microscope, and the ability to control the outcome when used as a nano-manipulator, depend on the size, shape, chemical composition and surface properties of the tip. But in the vast majority of experiments done today, the tip is a very crude instrument. STM tips are usually metal wires etched to a sharp point or simply snapped off with a pair of scissors. SFM tips are most often microfabricated pyramids of silicon or silicon nitride. In either case, the end of the probe is at least tens and usually hundreds of ångströms across. The STM and SFM can achieve high reso-

lution with such blunt tips because they sometimes have well-placed, atomic-scale protrusions, and it is these 'asperities' that make the primary contact with the sample. Unfortunately, even when such protrusions exist, their size, shape and chemical composition are completely unknown, and frequently change during



Tipped off — a carbon nanotube (some 10 nm in diameter and 100 nm long) stuck on the end of a conventional silicon probe used in scanning force microscopy. The probe and its associated cantilever arm are made of silicon. The nanotube tip is attached by coating the larger probe with glue and then bringing it in contact with a bundle of nanotubes; on pulling the probe away, a single tube can be left stuck on the probe's end.

the course of an experiment.

Needless to say, the lack of reliable probe tips is a serious impediment to progress in either nano-manipulation or straight microscopy. The ideal probe would be sharp (from a few ångströms to a few nanometres), with a well-defined, reproducible geometry on the atomic scale. It would also be chemically inert, conductive (for STM experiments or electrically stimulated manipulation experiments), easy to build, and cheap. For SFM experiments with biomolecules, it should be hydrophobic.

Virtually all of this wish list is satisfied by the new probe reported by Dai *et al.*¹. Fabrication of the probe is simplicity itself: a glue-coated SFM or STM tip is brought up to a preparation of multiwalled fullerene nanotubes, touched to a nanotube bundle, and then pulled away. Under favourable circumstances the bundle separates from the other nanotubes leaving a single, slender tube, with a diameter of between 5 and 20 nm and length of up to several micrometres, extending from one end. High-resolution electron micrographs show that the nanotube end is usually closed by an inert, hydrophobic fullerene cage. Single-walled nanotubes, with diameters of only 14 Å and capped by a hemispherical fullerene dome, can also be attached.

Somewhat surprisingly, such tips are strong enough to function as SFM probes, and even survive catastrophic crashes with

the surface, apparently by flexing instead of breaking as a normal tip would. This flexing also limits the maximum force that can be applied to the sample, so nanotube tips have a built-in 'soft-touch' feature that should make them preferable for use on delicate samples that might be damaged by stiffer conventional tips. As a final bonus, nanotubes are conductive, and so can be used as STM tips, or, in SFM experiments, to measure electrochemical currents or to modify samples by applying voltage pulses. For example, Dai *et al.* use

a voltage pulse to create a 40-nm dot on a gold surface in an SFM experiment, and also use a nanotube tip in an atomic-resolution STM experiment on a tantalum disulphide surface.

This is the first reported example of what might be called a 'molecular tool': a single, well-defined molecular structure connected to a macroscopic control device. Three features of the fullerene nanotubes combine to make such a microscopic tool possible. First, the nanotubes themselves are small and self-assembling, and their natural shape is nearly ideal for an STM or SFM probe. Second, they occur in large bundles with dimensions on the micrometre scale, which can be viewed with a light microscope and handled with micromanipulators. Finally, and somewhat fortuitously, the large bundles break apart in a way that leaves single tubes exposed.

The idea of using a fullerene as a probe tip for the SFM or STM has been bandied about in the probe microscope community for years, but has not been realized until now. The new nanotube tips give us the first opportunity to probe molecular-scale samples with a known molecular structure, and thus to reliably control the tip-sample interaction. The immediate benefit is likely to be an increase in the resolution and reliability of SFM experiments, where the need for good probe tips is acute. Further refinement of the basic idea (perhaps by chemically modifying the tip end, or by adding layers of insulation to the nanotube, or by attaching other molecules) should make many more such molecular tools possible, and open new avenues of research in which the properties of matter can be measured and manipulated directly, one atom at a time. □

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Computing with cyclic AMP

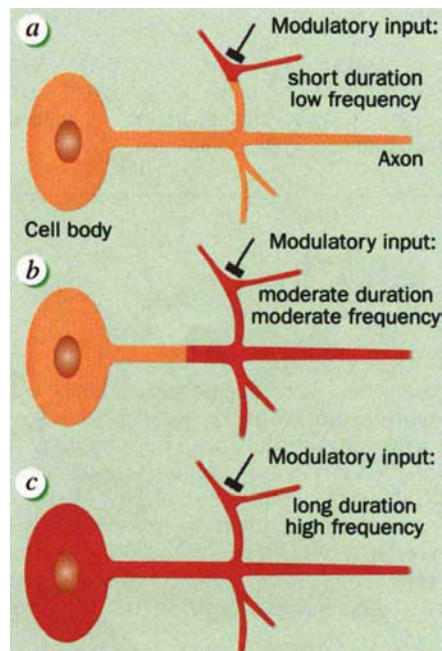
Eve Marder

It is immensely satisfying to be able to look directly at a well-known biological phenomenon. Nervous systems, whether large or small, employ neuromodulatory substances to fine-tune synapses, neuronal excitability and circuit dynamics, to produce a range of behaviourally relevant outputs^{1,2}. On page 166 of this issue, Hempel *et al.*³ visualize the spatial and temporal distribution of cyclic AMP in response to neuromodulators and modulatory inputs in the stomatogastric nervous system of the lobster *Panulirus interruptus*. The spatio-temporal dynamics of the increased concentrations of cAMP that arise from synaptic modulation of this circuit have interesting implications for our understanding of how events that trigger local signal-transduction pathways in synaptic regions lead to more distributed, or even global, regulation of neuronal properties.

As an intracellular messenger, cAMP mediates many important physiological responses, such as ion-channel function and gene expression. Levels of cAMP can be visualized directly⁴, and this approach has been used to investigate the dynamics of cAMP movement in living cells^{4,5}. The catalytic and regulatory subunits of cAMP-dependent protein kinase are labelled with fluorescein and rhodamine, respectively, and the enzyme is injected into the cell. The holoenzyme complex shows fluorescence energy transfer; however, this is eliminated when the regulatory and catalytic subunits dissociate in response to cAMP binding. By ratiometric imaging using a confocal microscope, the concentration of cAMP can be determined⁴.

This method has previously been used to study the effects of serotonin on cultured neurons from the mollusc *Aplysia californica*⁵. In response to bath application of serotonin, an elevated concentration of cAMP was first observed in the small processes, and then within several minutes, increased levels of cAMP were seen over most of the neuron. The timescale for the spread of the elevated cAMP is consistent with its rapid diffusion rate — within 20 seconds of a cAMP injection, elevated levels of cAMP had spread almost 200 μm . Bacskaï *et al.*⁵ have also shown that the free catalytic subunit of the cAMP-dependent protein kinase translocates in and then out of the nucleus on a timescale of about two hours in response to long-lasting serotonin applications.

Now Hempel *et al.* extend these observations to the synaptic release of modulatory substances from neural inputs to an intact and functioning circuit. They first physiologically identified the cell bodies of the stomatogastric ganglion, which is a popular model system for the study of



The spatial extent of changes in the concentration of cAMP (red) will depend on the temporal dynamics of local modulator release. Hempel *et al.*³ show that, in response to nerve stimulation, the first increase in cAMP is seen in fine neuropilar processes. The time course of recovery of cAMP levels after stimulation is long enough for levels of cAMP to slowly build up and spread in response to repeated short stimuli, or in response to longer trains of activity. *a*, Stimuli of short duration and low frequency produce only a local increase in cAMP; *b*, stimuli of moderate duration and frequency evoke the build-up and spread of cAMP through a much larger region of the neuron if the decline in the concentration of cAMP is slow relative to the frequency of the stimulus; *c*, stimuli of long duration or high frequency lead to the spread of cAMP through the entire neuron (including the cell body).

rhythmic motor patterns and contains a small number of neurons that can be individually identified. They then looked at changes in the levels of cAMP after applying several of the modulatory substances that are known to activate the pyloric and gastric-mill rhythms produced by the stomatogastric nervous system⁶. Each modulator increased the levels of cAMP in a different subset of neurons within the stomatogastric ganglion. This is not surprising given that each modulator produced different patterns of activity in the ganglion^{2,6}. But earlier studies had addressed the effects of these same modulators on cAMP levels in whole ganglion extracts, and they had found that octopamine produced the largest increase in cAMP⁷. By contrast, Hempel *et al.* showed that octopamine modestly increased the levels of cAMP in many neu-

rons, whereas dopamine and serotonin activated fewer neurons more strongly. This reminds us that biochemical studies done on whole ganglia or parts of the brain are always average measurements that will often underestimate an important effect that is shown by only a few neurons.

Hempel *et al.* also measured changes in the levels of cAMP in individual neurons after stimulation of neuromodulatory inputs to the stomatogastric ganglion. They stimulated the stomatogastric nerve, which contains the axons of many modulatory projection fibres containing a large number of neurotransmitters and neuropeptides⁸. In contrast to uniform bath application, they used the normal presynaptic delivery, so the changes in cAMP levels that were evoked by the physiological liberation of neurally released modulatory substances could be tracked. In a beautiful image, they showed the spatial and temporal spread of the elevated levels of cAMP in an identified PY neuron (one of the motor neurons of the stomatogastric ganglion).

In response to a 30-second stimulation of the stomatogastric nerve, the fine processes in the neuropil, where synaptic contacts are found, showed a cAMP signal within a few seconds; this increased throughout the duration of the stimulation before levelling off. In response to a 60-second stimulation, cAMP levels rose in the larger processes of the cell, and there was a delay that was consistent with the diffusion of cAMP from the smaller processes. A 3-minute stimulation evoked an increase in cAMP in the cell body (several hundred micrometres from the small processes) that started with a 3–4-minute delay, again consistent with diffusion of cAMP throughout the neuron, from the fine processes to the cell body.

Because the authors could not image the whole neuron at once, we do not know the temporal evolution of the cAMP levels for the whole neuron during the 30-second, 60-second and 3-minute stimuli. But the inference from these data is clear (see figure): trains of stimuli that are infrequent or of short duration release modulatory substances that are likely to produce local changes in cAMP levels. Such local changes could modulate ion channels and other processes within a restricted region of the neuron. Trains of

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stimuli that are of moderate frequency or duration may alter the levels of cAMP throughout a large region of the neuron; however, they probably produce only limited changes in the cell body. But prolonged or repeated synaptic activation can result in elevated levels of cAMP in the cell body, and produce a series of cAMP-regulated transcriptional events^{9,10}.

The time course of decay of the cAMP signal is long and the spread of the signal

large, so intracellular levels of cAMP could serve as a temporal and spatial integrator⁵. In other words, intracellular cAMP levels can be viewed as a 'memory trace in time and space' of the previous history of synaptic activation. □

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GEOMETRY

Pulling the knot tight

H. K. Moffatt

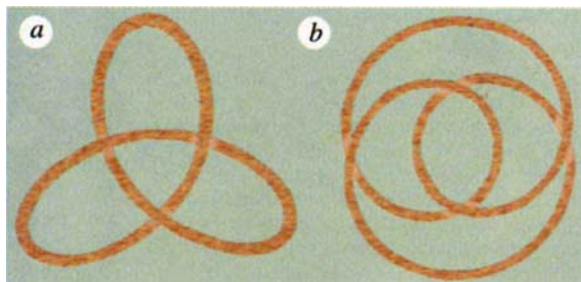
If a complicated knot in a piece of string is pulled tight, it seems likely that it should always achieve roughly the same geometrical configuration, with a minimum length of string within the knot. If the string is strongly twisted first, the tightening processes may result in an unpleasant tangle; but otherwise, as every boy or girl scout knows, the operation can proceed with a predictable and repeatable result at every trial. How, then, can the 'ideal' ultimate configuration of a knot be defined and determined? Vsevolod Katritch *et al.* on page 142 of this issue¹ (see also Andrzej Stasiak *et al.*² on page 122) have found an ingenious approach to this problem. The resulting ideal configurations are remarkably similar to equilibrium configurations of knotted DNA molecules, so in some sense it would appear that DNA chains are pulled tight, and that they do not like to adopt highly convoluted configurations.

Knots are usually treated as closed curves, by simply joining the loose ends of the piece of string. The knot is then characterized by the topology of this curve, two knots being topologically equivalent if one can be obtained from the other by continuous deformation of the piece of string. Thus, the trefoil knot $T_{3,2}$ is topologically equivalent to the trefoil knot $T_{2,3}$ (see figure), but the granny knot is not equivalent to the reef knot.

Given two complicated knots, it may be extremely difficult to decide whether they are topologically equivalent (the same knot) or not. So mathematicians have developed increasingly sophisticated techniques for discriminating between them. For example, for every knot, one can construct an Alexander polynomial³ based on the knot crossings when viewed in any plane projection, and this polynomial is invariant under any continuous deformation of the knot. Thus, two knots having

different Alexander polynomials are certainly distinct. More subtle discrimination is provided by the Jones polynomial⁴, but as yet there is no complete classification of knots of very high complexity.

Suppose now that we have a knotted closed curve C , and that we place around it a tube of small constant radius r so that the curve becomes a string. If, for a given



The trefoil knot, in different configurations $T_{2,3}$ (a) and $T_{3,2}$ (b). The trefoil is a special case of the torus knot $T_{n,m}$ which winds n times round a torus the long way, and m times the short way.

configuration of C , r is gradually increased (the tube is inflated), then we mimic the process of knot tightening. There are two limiting factors in this process: first, r cannot increase above the minimum radius of curvature of C , as otherwise the tube would locally intersect itself; second, more remote parts of the tube may come into contact with one another. If the initial configuration of C is varied, keeping its length constant, then the maximum value of r will also vary. Katritch *et al.*¹ have devised a numerical technique whereby $r_{\max}$ may be systematically increased; the corresponding configuration of C is then described as 'ideal'.

This technique is closely related to a different one involving the concept of 'knot energy'^{5,6}. The knot is identified with an imaginary magnetic flux tube, which is allowed to contract in response to Maxwell tension, with a corresponding increase in cross-section to maintain total tube volume. The process of contraction is again arrested when the tube comes into contact with itself, and a minimum-energy equilibrium state is attained. The process is dis-

tinct from that envisaged by Katritch and colleagues in that the tube cross-section may, and in general does, deform from a circle during the final stage of contraction. But one may seek minimum-energy states within the family of constrained uniform tube configurations⁷, and then the similarity with the purely geometrical construction of Katritch *et al.* becomes more apparent.

In the previous work, it was by no means clear that the minimum-energy configuration for a given knot is uniquely determined. It seems likely that the trefoil knot, for example, may end up in either the $T_{2,3}$ or $T_{3,2}$ configuration, each at a local energy minimum. By contrast, Katritch *et al.* have found that every knot investigated 'flows' to a uniquely determined ideal configuration¹. Even the Perko pair, two different representations of a knot of ten crossings that were thought to be distinct until 1974, are shown to flow to the same ideal configuration. It would be interesting to test the algorithm on the simpler $T_{3,2}$ and $T_{2,3}$ configurations of the trefoil; it is not clear to me how $T_{3,2}$ could flow to $T_{2,3}$ through the process described.

Two properties of the ideal configuration appear to have physical significance: crossing number and writhe. The averaged crossing number has been shown by Freedman and He⁶ to provide a lower bound on knot energy. Katritch and colleagues demonstrate a remarkable linear relationship between the length-to-diameter ratio in the ideal configuration and the average crossing number, which provides a useful measure of knot complexity.

But it is in modelling equilibrium configurations of knotted DNA molecules that this work is at its most thought-provoking. Averaging over an ensemble of millions of configurations for each knot type, the authors found that the mean writhe (an average over all projections of the number of crossings, with positive and negative signs according to handedness) is very close to that of the ideal configuration, and the mean average crossing number is linearly related to that of the ideal configuration. This is for molecules that are 'nicked' (having no torsional stress), a condition that must have a bearing on the writhe. One is led to speculate that the DNA molecule seeks to attain a minimum energy state. But what precisely is the energy that is minimized? □

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Brain waves deciphered

John P. Miller

THE expression 'brain waves' has been adopted into our colloquial vocabulary, and conjures up a sense of mystery in matters related to mind and brain. And rightly so — although waves of electrical activity in recordings from mammalian brains were first seen more than 120 years ago¹, the physiological significance of these oscillations remains a matter of conjecture. On page 162 of this issue, Wehr and Laurent² take us a step closer towards understanding the functional relevance of brain waves. Specifically, they show how a considerable amount of information about sensory stimuli is encoded by the sequences of oscillatory activity within groups of nerve cells.

What we commonly refer to as brain waves are electrical oscillations that emerge from coordinated electrical activity across large groups of neurons³. Such oscillatory activity is a general property of neural systems in many animal species, ranging from snails⁴ to humans⁵. In most areas of the human brain, recordings from single cells show little or no overt evidence of oscillatory activity. But when an electroencephalogram is taken by placing a button-sized probe on a person's scalp, oscillations at several characteristic frequencies are clearly visible. They arise from a synchronized rhythmic electrical activity across large groups of neurons.

To illustrate, consider a group of a thousand nerve cells in an olfactory centre of the brain, all of which are responsive to the same olfactory stimulus — say, the smell of a rose. Assume that the presentation of this odour elicits a uniformly spaced sequence of approximately 40 action potentials over a period of one second in each of those cells. What would the combined activity of all of the cells look like when recorded with a large-scale electrode? If the cells were not synchronized with one another, then the 40,000 action potentials that they generated would be distributed fairly uniformly within the one-second response interval. In this case, the combined activity would form a 'flat', featureless signal at a scalp electrode. However, if all of those cells fired their action potentials in near-synchrony, the 40,000 action potentials would be organized into 40 groups of 1,000 events during the one-second interval. Summation of this pattern would result in wave-like oscillations at 40 Hz.

This stimulus-evoked oscillatory synchronization is reasonably well understood in many cases, yet we still do not know the significance of these oscillations within the context of brain function. Do any details of the synchronized oscillations encode significant information, or are the oscillations merely epiphenomena

of the complex mass-activity of nervous systems? If the oscillations are involved in encoding information, what is that information, and what is the nature of the 'neural code' through which the information is represented?

These are precisely the types of questions being addressed by Laurent and colleagues. By studying olfactory coding in the antennal lobe system in the locust brain, they previously found that, when a locust is presented with certain odours, oscillatory waves of activity are generated in the antennal lobe at frequencies of about 20 Hz (refs 6, 7). These oscillatory patterns are strikingly similar to the waves of activity that are generated at the equivalent processing stage in mammalian olfactory systems³. By recording the activity in both single neurons and whole neural networks, they were able to show that each odour leads to the synchronous activation of a specific ensemble of neurons within the antennal lobe. Each 'assembly' comprises about 100 of the roughly 800-strong class of cells known as the projection neurons.

Laurent and colleagues went on to pre-

dict that different odours could be encoded by the subset of these neurons that is involved in the electrical activity elicited by the odour. Each individual projection neuron could belong to many different assemblies and participate in the coding for many different odours. This corresponds to a 'spatial combinatorial code', whereby each odour elicits the activation of a different spatial pattern of 100 neurons within the 800-cell array. The unique assembly of cells that are stimulated by a particular odour could be identified by their synchronous firing, so the observed oscillations are a direct correlate of this functional 'tag'.

Wehr and Laurent² have now extended the hypothesized combinatorial encoding scheme to the temporal domain. They show that the set of neurons that is activated during the presentation of any particular odour changes in a reliable, stimulus-specific manner. Although around 100 projection neurons are activated during the presentation of any odour, only a small subset of these is maximally active during any single cycle of the oscillations. In other words, each 100-cell assembly is made up of several sub-assemblies, each of which has a different time course for its contribution to the overall response pattern. So whereas one cell might fire with high probability during the

PALAEONTOLOGY

Fossil fortune cookie

CATHAYMYRUS diadexus, pictured here (and described by Shu *et al.* on page 157 of this issue) is a fortune cookie. Of the more than 10,000 fossils known from the 535-million-year-old Chengjiang fauna of China, *Cathaymyrus* is represented by just one. The trivial name *diadexus* comes from the Greek for a harbinger of good fortune, in the explicit hope that more specimens turn up.

Cathaymyrus joins select company — the scarce and contentious fossils that convene around the base of the family tree of the chordates. This taxonomic rank includes all vertebrates and, for instance, the tunicates of tide pools and the lancelets (or amphioxus) of inshore waters. *Cathaymyrus* looks most like a lancelet, as does the better-known *Pikaia gracilens* from the celebrated Burgess Shales fauna of Canada, which is about ten million years younger than the Chengjiang fauna.

Debate springs eternal over the affinities of other forms, for example *Yunnanozoon*, also from the Chengjiang fauna. And the picture is muddled further by other strange fossils. Most (but not all) agree that the bizarre echinoderm-like fossils called calcichordates probably are not chordates; on the other hand, many (but not all) now concede

that the phosphatic tooth-like conodonts belonged to fully vertebrate chordates.

The diversity of chordates in the past was probably much greater than the Recent fauna gives us cause to believe. This is why fossils claimed to have chordate affinity will always seem unusual, and generate controversy. If only the fossil record were a little better: one hopes that the invocation of *Cathaymyrus diadexus* has the desired effects.

Henry Gee

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Dudley Simons & Les Goodey

first three cycles, another might fire only during the second to the fifth cycles, and so on. Each odour would elicit the activation of a different sequence of spatial patterns of about 100 projection neuron cells. This spatio-temporal combinatorial coding scheme is likely to be much more efficient than a spatial coding scheme, because each set of sub-assemblies could be activated in different temporal sequences to indicate different odours. But here again, oscillations are direct correlates of the functional tags for specific odours.

In a related study⁸, MacLeod and Laurent investigated the physiological mechanisms that underlie inter-cell synchronization. They were able to abolish the synchronization in the odour-coding assemblies without affecting the temporal response properties of the individual cells. This was done by blocking inhibitory synapses onto the projection neurons by another class of neurons within the olfactory network. These results support theoretical models that have been proposed for several other systems — including the mammalian olfactory cortex⁹ —

which depend on inhibitory interactions to achieve oscillatory synchronization.

Determination of the neural coding schemes that are at work in nervous systems is an important goal. Not only is the nature of neural coding of great interest, but a knowledge of the coding schemes will be necessary for the development of theories about cortical function and neural computation. Through their work on the locust olfactory system, Wehr and Laurent have taken us closer to achieving this aim. □

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HIV

One on one meets two

Simon Wain-Hobson

VIRUSES identify their target cells by recognizing protein or carbohydrate molecules on the cell surface. Once the virus has docked, subsequent manoeuvres allow it to enter the cell and nucleic-acid replication ensues. More than ten years ago, the CD4 molecule was found to be the receptor for human immunodeficiency virus^{1,2}. But it was quickly shown that CD4 was not enough, and the hunt began for the second receptor. Despite the use of some awesome firepower to track down the missing receptor, it was only earlier this year that it was identified as a transmembrane, G-protein-associated molecule called CXCR-4 (ref. 3). Since then, the field has moved quickly — there is now known to be another HIV co-receptor, termed CCR-5, which is related to CXCR-4. Most HIV isolates use CCR-5 as the second receptor^{4,5}, and two papers by Wu *et al.*⁶ and Trkola *et al.*⁷ on pages 179 and 184 of this issue now provide a possible mechanism by which the CCR-5 receptor could mediate the entry of HIV into the host cell.

HIV encodes a glycoprotein precursor called gp160 that is cleaved by cellular enzymes into gp120 and gp41, which are surface and transmembrane proteins, respectively: gp120 is non-covalently attached to gp41, which is anchored in the viral lipid bilayer (see figure). High-affinity binding of gp120 to the first HIV receptor, CD4, causes conformational changes in gp120. These can be detected by the occlu-

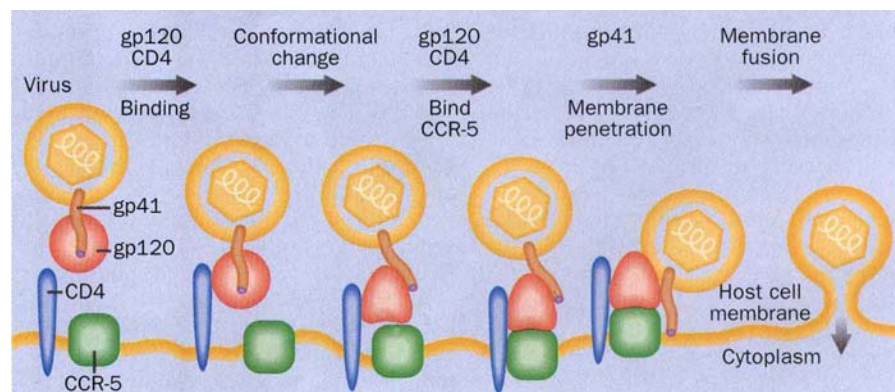
sion of some epitopes for monoclonal antibodies. The results of Wu *et al.* and Trkola *et al.* indicate that these conformational changes lead to the creation of a new recognition site on gp120 for CCR-5, to which it binds with nanomolar affinity. The two groups used a simple biochemical approach to show this previously unsuspected interaction. CCR-5 acts as a receptor for a class of molecules known as β -chemokines; for example, macrophage inflammatory protein (MIP)-1 α and -1 β and RANTES (regulation-upon-activation normal T expressed and secreted). By

using radioactively labelled MIP-1 β , both groups were able to show that the binding of MIP-1 β to CCR-5 could be blocked by the gp120/CD4 complex.

It will come as no surprise to HIV aficionados that the V3 loop of gp120, something akin to a molecular Achilles heel, is involved in these interactions. Not only is the V3 loop the target of neutralizing antibody, but it is also involved in dictating the use of CCR-5 or CXCR-4. The part played by V3 was elegantly shown by using gp120 derivatives in which V3 had been deleted — and there was no longer an interaction between gp120 and CCR-5.

Wu *et al.* and Trkola *et al.* also found that many of the monoclonal antibodies that can neutralize HIV in a simple culture assay⁸ blocked the binding of gp120/CD4 to CCR-5. This is a satisfying finding given the difficulties of working with gp120, which is a large, heavily glycosylated, polymorphic molecule that has defied many attempts to crystallize it. Moreover, neutralizing antibodies often block viral infection by smothering the regions that are involved in receptor binding. By using two receptor molecules, one might have thought that HIV had made life difficult for itself, allowing the immune system to strike twice. But compared with many other viruses, anti-HIV neutralizing-antibody titres are low, indicating that HIV has found a robust solution to coping with the wrath of the immune system.

Yet there is still something missing. Studies of many enveloped viruses indicate that the hydrophobic amino terminus of the transmembrane protein (that is, gp41 in HIV) must somehow interact with the membrane of the host cell. This situation has been well characterized in influenza virus: the receptor-binding envelope protein is called haemagglutinin, and it is analogous to HIV gp160. In the same way as gp160 is cleaved into gp120 and gp41, haemagglutinin is cleaved into HA1 and HA2. The crystal structure of haemagglutinin shows that the amino terminus is



HIV-encoded gp120 recognizes the host-encoded CD4 receptor. This interaction leads to a conformational change in gp120, allowing it to bind to a second receptor, CCR-5, as shown by Wu *et al.*⁶ and Trkola *et al.*⁷ At some point to be defined, the amino terminus of gp41 is uncovered, allowing penetration of the host cell membrane and fusion of the viral and host cell membranes. Stripped of its lipid protection, the capsid complex moves into the cytoplasm, and reverse transcription is initiated depending on the availability of nucleoside triphosphates.

buried deep within the HA1/HA2 complex. During viral infection, HA1 binds to a carbohydrate moiety on the surface of the target cell, and this is followed by endocytosis of the virus. Exposure to acid in the lysosomal compartment of the target cell leads to the extensive reorganization of HA2 to form a spectacular coiled coil⁹. The amino terminus of HA2 is now thought to be free to penetrate the host cell membrane.

By contrast, pH changes are not involved in HIV infection, and the gp41 molecule does not seem to interact with CCR-5. Yet some of the properties of the extracellular domain of gp41 alone are similar to those of the reorganized low-pH form of HA2 (ref. 10). So could the binding of CCR-5 also cause extensive changes to gp41, thereby freeing up its amino terminus? At the current rate of progress, we probably won't have long to wait for an answer to this question. Might there even be a third receptor? This is unlikely because, when both the CCR-5 and CD4 genes are transfected into mouse cells, the cells can be infected by HIV.

It has been known for some time that certain CD4-negative cell lines may be infected with HIV if large concentrations of the virus are used¹¹. In view of this, the interaction between CCR-5 and gp120, described by Wu *et al.* and Trkola *et al.*, is intriguing. It is possible that the subtle genetic complexity inherent in a high-titre virus stock is enough to allow the selection of variants that are better adapted to life in the absence of CD4. Might HIV forgo the use of CD4 *in vivo*? Given the high affinity of gp120 for CD4 and the huge number of CD4-positive T cells in the body ($>10^9$, even for AIDS patients), this is unlikely. However, in late-stage disease, when viral load is at its greatest, CD8-positive T lymphocytes may be heavily infected by HIV-1 (ref. 12) — any guesses as to how the virus got in?

The near future will see the molecular mapping of the interaction between gp120, CD4 and CCR-5 and, we can hope, an understanding of the detailed mechanics of gp41-mediated virus–host membrane fusion. But whatever discoveries lie around the corner, we can be sure that it will be some time before we unravel all of the complex interactions involved in the infection of a target host cell by HIV. □

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Harmonics to quicken the pulse

Paul Corkum

EXPERIMENTAL science now stands on the verge of combining spatial and temporal measurement on an atomic scale of precision — that is, with both ångström (10^{-10} m) and attosecond (10^{-18} s) resolution. Two of the most recent advances are the development of femtosecond (10^{-15} s) X-ray pulses, described by Schoenlein *et al.* in *Science*¹, and the calculation of the sub-femtosecond characteristics of X-rays produced by high-harmonic generation, as described by Antoine *et al.* in *Physical Review Letters*².

With the science and technology of coherent X-ray generation rapidly becoming

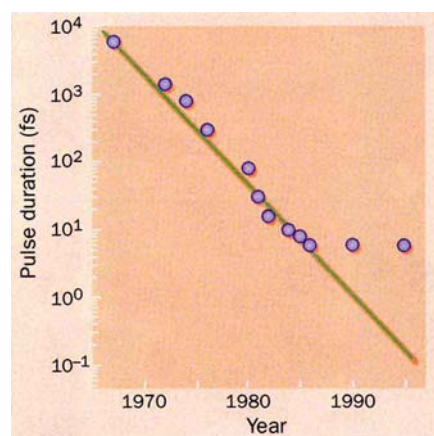
photons and the number of electrons. With better electron focusing and a shorter electron bunch, the output can be increased in future experiments and the pulse duration decreased.

In a second approach, represented by the work of Antoine *et al.*², the high photon density in femtosecond optical pulses is used. When these intense ultra-short pulses irradiate atomic gases with a high ionization potential, a series of odd harmonics is emitted with almost constant power in each one, up to a maximum harmonic (even harmonics are forbidden by symmetry in a symmetric medium). With modern titanium–sapphire lasers, these harmonics can stretch from the third to beyond the 100th harmonic. Radiation with energies near 200 eV has been generated in several labs, and reaching the 40-Å (300 eV) 'water window' for probing live biological samples is not out of the question.

Antoine and co-workers have now shown theoretically that if we look at a set of harmonics together, then the output pulse consists of a train of sub-pulses, each separated from the next by half the period of the fundamental (that is, the pumping laser frequency). For the experimentally important example of harmonics produced by a titanium–sapphire laser, the duration of the sub-pulses can be as short as 100 attoseconds, and the separation between them will be about 1.5 femtoseconds. Many laboratories around the world probably routinely produce trains of attosecond pulses like those described in this paper, but because no methods for observing them exist yet, they are unaware of it.

Attosecond pulse trains have a high peak power. Experiments show that each harmonic in the 50-eV range can contain as many as 10^9 photons (ref. 3), so the radiation of 10 adjacent harmonics can consist of 10^{10} photons. If that radiation is contained in 50 sub-pulses each of 100 attoseconds, then the peak power is 16 megawatts. If focused to a diameter of one micrometre, the intensity would exceed 1×10^{19} W m⁻², or 10^{22} photons m⁻². Such intense sources at such short wavelengths have never been available before.

What might they be used for? X-ray pulses are commonly used to determine the structure of molecules or solids. The new femtosecond sources have unique features, such as their accurate synchronization with a high-power visible or infrared laser. One exciting possibility is initiating a chemical, physical or biological process with a femtosecond visible or infrared pulse and following structural



Optical pulse duration, in femtoseconds, plotted against the year in which new speed records were set. The line shows the exponential fall of pulse duration until about 1986. If single 100-attosecond pulses are achieved in the next few years, the historical trend will be regained.

ing a forefront of optical research, it is important to understand the major technological trends and their implications. The tools that make these ultra-short-pulse X-ray sources are ultra-short-pulse lasers themselves. Ultra-short pulses are unique in achieving high photon densities with only moderate numbers of photons. This characteristic has been used in two different ways.

In one approach, Schoenlein and colleagues¹ produce sub-ångström radiation by scattering the visible pulse off a beam of high-energy (100 MeV) electrons. The frequency of the scattered photons is increased by a factor proportional to γ^2 (where γ is the Lorentz factor of the electrons, here about 200), producing a 300-femtosecond pulse containing 5×10^4 photons with a wavelength of 0.4 Å. The output pulse duration is determined by the time it takes the optical pulse to travel from one side of the electron beam to the other, and the number of scattered X-rays is proportional to the number of optical

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changes with high precision using an X-ray pulse. For some applications, X-rays will have to compete with at least two other approaches to time-resolved scattering experiments: femtosecond electron diffraction and ion scattering from Coulomb explosion initiated by intense ultra-short pulses.

Ultra-short X-ray pulses can serve still other purposes. The work of Antoine and colleagues implies that X-ray nonlinear optics is also within reach. The single-photon cross-section for ionization of helium with 50-eV photons is $2 \times 10^{-22} \text{ m}^2$, and Antoine *et al.* predict that pulses can deliver about 10^{22} photons per square metre, enough to ionize most of the helium atoms in a sample. Saturation is one of the simplest processes in nonlinear optics, and the possibility of saturated ionization implies that other nonlinear processes should be possible too. If a single 100-attosecond pulse can be selected, a prospect that appears to be within reach, it should also be possible to excite a sample (a metal, for example) with one pulse and then to probe its response with a second.

No matter what the technology, it seems clear that the dream of being able to follow time-dependent molecular and atomic structure is within reach.

The next step must be the development

of methods of measurement, so that attosecond pulses can be recognized when they are produced. The first attempts will probably use atomic ionization, because if ionization is fast enough to produce attosecond pulses, it should be fast enough to permit their measurement. Ultimately, however, attosecond technology will probably rely on nonlinear X-ray optics just as femtosecond technology now relies on nonlinear optical techniques.

The progress in ultra-fast X-ray technology represented by these two papers seems likely to accelerate, and the trend of optical physics to generating ever-shorter pulses (see figure) may be regained. The scientific research community as a whole should be aware of the emerging opportunities, and it is not too early for biologists, chemists and physicists to be considering the questions that this new technology can help to solve. □

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DEVELOPMENTAL BIOLOGY

Patching up Hedgehog

Roel Nusse

THE ability of cells to respond to activating signals during development is primarily determined by specific receptors on their surfaces. Given the widespread developmental roles of the Hedgehog (Hh) family of signalling molecules, the identification of receptors for these proteins has been high on the agenda. On pages 129 and 176 of this issue^{1,2}, two groups now provide strong support for a hypothesis on Hh signalling that was formulated a number of years ago³. Even before the molecular characterization of *hh*, Phil Ingham proposed that the multiple-pass transmembrane protein Patched (Ptc) would act as the Hh receptor. This model fell out of favour when another *Drosophila* segment-polarity gene, *smoothed* (*smo*), became a leading contender for being the Hh receptor^{4,5}. But it now seems that the original proposal was right — Ptc is the primary receptor for Hh.

The *Drosophila* *hh* gene has been implicated in the segmentation of embryos and in patterning of imaginal-disk outgrowth. In vertebrates, the *hh* gene family controls important developmental decisions such as the polarity of limb outgrowth, floor-

plate induction in the spinal cord and somite patterning.

The phenotypes of the segment-polarity genes in *Drosophila* provided the initial hints for interactions between their

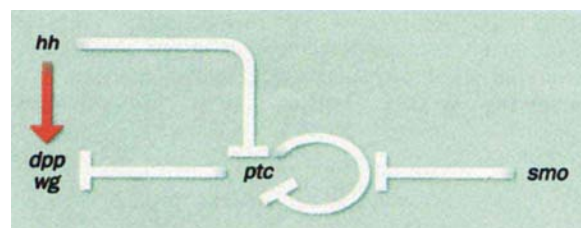


FIG. 1 Outline of interactions between genes in the *hedgehog* pathway. Although *hh* is needed for the expression of *decapentaplegic* and *wingless*, its main function is thought to be the repression of the *patched* gene, which can also be repressed by *smoothed*.

gene products (Fig. 1), although some of these clues were perhaps counter-intuitive because of intermingled negative and positive interactions. For example, *hh* is required for the expression of downstream genes such as *wingless* (*wg*) and *decapentaplegic* (*dpp*), whereas *ptc* has the opposite function: it represses *wg* and *dpp*. So *dpp* and *wg* become

ectopically expressed in the absence of functional Ptc. The *ptc* gene also represses its own function, leading to high expression of inactivated *ptc* — another counter-intuitive situation. Hh is a secreted protein that inactivates Ptc, leading to spatially restricted expression of *wg*, *dpp* and *ptc*. A *hhptc* double mutant has a similar phenotype to *ptc* single mutants³, indicating that in the absence of *ptc*, *hh* is not necessary. This supports the proposal that the main function of *hh* is to suppress *ptc*³.

In contrast to *ptc*, *smo* mutants display similar phenotypes to *hh* mutants, both in embryos and in clones of mutant cells in *Drosophila* imaginal disks. Moreover, the effects of ectopic Hh become negligible when cells lack *smo*. Smo is a seven-pass transmembrane protein that has homology to members of the *frizzled* (*fz*) gene family. When embryos lack both *smo* and *ptc*, they have a similar phenotype to *smo* single mutants, indicating that *ptc* is located genetically upstream of *smo*^{4–6}.

To establish a biochemical basis for the interactions between these genes, Stone *et al.*¹ have transfected mammalian cell lines with *ptc*, and Marigo *et al.*² have used *Xenopus* oocytes to express Ptc that is produced from injected messenger RNA. Both groups find that labelled Hh protein binds to the Ptc-expressing cells with high affinity, and Hh and Ptc can also be co-immunoprecipitated and crosslinked. The binding constant is within the same range as the concentration of Hh protein at which it is biologically active in various *in vitro* assays⁷. Chen and Struhl⁶ have shown that clones of cells that lack Ptc become permeable to the Hh signal. Cells that border the far end of the clone can respond to Hh, which would not be able to traverse this distance if confronted with cells that were expressing *ptc*. This suggests that Ptc soaks up the Hh signal, acting as a sink and as a receptor. As the expression of *ptc* is controlled by Hh signalling, the abundance of Ptc seems to limit the spread of the very same signal that leads to its increase⁶.

So how is *smo* involved in the Hh pathway? Stone *et al.*¹ show that Smo does not interact directly with Hh, as cells that express *smo* fail to bind Hh. But they make the

intriguing observation that when Ptc and Smo are coexpressed, the two proteins form a complex that can be detected by co-immunoprecipitation. With the caveat that interactions between Ptc and Smo have yet to be seen in cells that contain normal levels of these proteins, these data indicate that Ptc and Smo may form a heteromeric signalling complex in which Ptc

initially binds the ligand Hh, and Smo transduces the signal. The affinity of Hh for Ptc does not change when Smo is also expressed, arguing against a dimeric higher-affinity receptor. The available genetic and biochemical data imply that unoccupied Ptc inactivates the Smo pro-

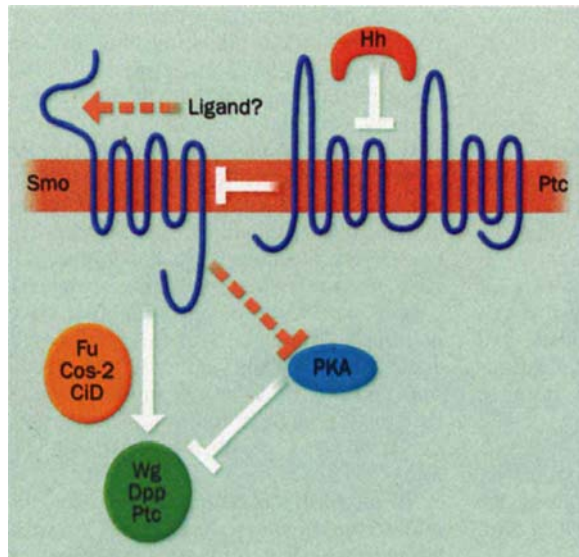


FIG. 2 Interactions between Hh and components of its signalling pathway, as shown by Stone *et al.*¹ and Marigo *et al.*². The secreted Hh protein binds to Ptc, which spans the membrane 12 times. This binding relieves the Ptc-dependent inhibition of Smo, and may involve direct contact between Smo and Ptc. Once it has been relieved from inhibitory signals, Smo activates the downstream genes *wg*, *dpp* and *ptc*, through the signalling components Fused (Fu), Costal-2 (Cos-2) and Cubitus interruptus (CiD). This may work through the inhibition of protein kinase A (PKA), although it is most likely that PKA is not regulated by Hh signalling, and that it acts in parallel. Dashed arrows indicate uncertain interactions, other arrows the more trusted ones.

tein, and the binding of Hh to Ptc relieves this inhibition, thereby activating Smo (Fig. 2). In this case, Smo would have a constitutive (that is, ligand-independent) activity in the absence of any inhibition by Ptc. But it seems equally possible that Smo has a ligand of its own, the product of a gene that has not been uncovered in genetic screens. Given the similarity between Smo and the Fz proteins, such a ligand may be a Wg-related protein, as these secreted molecules can use Fz proteins as receptors⁸.

To test these models further, one would need to measure the downstream effects of Hh in cell-culture assays, in a receptor-dependent manner. Fortunately, there are a number of additional genes in the Hh pathway, some of which have measurable enzymatic functions. For example, *Drosophila* S2 cells (which normally express *ptc*) respond to the addition of Hh by phosphorylating Fused, which is itself a protein kinase⁹. A better-known protein kinase in the Hh pathway is protein kinase A (PKA; reviewed in ref. 10). Inactivation of PKA has the same effect as mutations in *ptc* — constitutive activity of the *hh* tar-

get genes. In a linear pathway model, the Hh receptor complex would inhibit the activity of PKA, unless the receptor itself was downregulated by ligand. Such a mechanism invokes cyclic AMP and a G protein, and this is an attractive candidate for a downstream effector of Smo. All too often, however, genetic interactions tend to be interpreted in a linear way, and there is good evidence that PKA acts in parallel to Hh/Ptc/Smo signalling (reviewed in ref. 10).

Finally, it is tempting to speculate how Hh interacts with its receptor. The Hh protein has many unusual properties, one of which is autocatalytic processing to generate a cholesterol-modified product¹¹. The addition of cholesterol may set constraints on the diffusion of Hh, by tethering it to the plasma membrane. The structure of the mature protein predicts that Hh has an enzymatic function; it may even be proteolytic¹². So Hh may work through a covalent modification of its receptor, although significant proteolytic processing of *ptc* is not detected⁷. Whatever the function of Hh, these and other stories show that the collection of segment-polarity genes is replete with intriguing molecules and mechanisms that are important for develop-

ment. And in view of the fact that human *ptc* is a tumour-suppressor gene, they may also turn out to be important for cancer^{13,14}.

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DAEDALUS

Algal transport

THOSE who dream of solar power always wake up to two big problems. First, you need a huge area of land to capture enough power, especially in cloudy climates. Second, you need some way of storing the energy for use at night. Daedalus now has a idea that solves both problems.

He recalls an old scheme to farm single-celled algae, notably *Chlorella*, which forms the green scum on stagnant ponds. *Chlorella* holds the record for photosynthetic efficiency, about 25%, and can be processed into a nutritious but unappetizing 'flour'. Daedalus wants to use it as a fuel for water transport. A canal could easily have 10,000 square metres of surface for every kilometre of its length. Over 24 hours it would intercept an average of perhaps 1 MW of solar power. Cover the surface with *Chlorella* scum, and the algae might capture and store 10% of this. A traditional canal barge was propelled on less than 1 kW (one horsepower, in fact). So each kilometre of the canal could power several such barges, even if their engines were not particularly efficient. Daedalus envisages some sort of rotating wire strainer ahead of the barge, to filter off the algal mat and feed it into a simple thermal engine. The waste heat from the engine could dry the incoming fuel.

In Britain and some European countries, canals were developed in the eighteenth century or earlier, when roads were sadly inadequate. In those unhygienic days, the canals often served as primitive sewage-disposal systems. Daedalus's algal canals could be used in the same way. The resulting eutrophication would recycle the waste into a thicker and more luxuriant algal scum. Furthermore, some plants are remarkably good at extracting heavy metals from solution. If *Chlorella* can be taught this trick too, industrial effluents could also be fed into the canals for reprocessing. Barge owners would salvage the ash from their engines to sell as metallic ore.

The other waste product from algal combustion is, of course, carbon dioxide. *Chlorella* grows much faster in water enriched with this gas, so it too could usefully be fed back into the canal. Daedalus freely admits that in a world awash with cheap fossil fuel and obsessed with powerful and dramatic forms of high-speed transport, his scheme is unattractive. It will come into its own in the next century, when fossil fuels have become scarce and expensive, global warming and high levels of carbon dioxide have boosted plant growth, and the rise in sea level has opened new canal routes.

David Jones

Kinematics of phalarope spinning

SIR — Phalaropes are wading birds that spin on the water, presumably to feed¹⁻⁵ because they peck while spinning. Recently, we demonstrated with high-speed photography that phalaropes do indeed feed while spinning⁶. These very small birds are the only vertebrates that spin. Larger birds probably could not spin fast enough to upwell prey. Spinning is energetically costly, about four times resting metabolism — phalaropes do not spin if adequate surface prey are available. Here we report the details of how these birds feed while spinning.

We first thought that phalaropes produce a bounded vortex with central

between 35° and 50°. The bird leans in and circles with a radius of half the water-line length, the bill above the upwelled core. The bird's body circles continuously, but its head moves in discrete 45° snaps, separated by brief pauses, like a spinning ballerina. Phalaropes detect prey, thrust, seize, transport and swallow in less than half a second⁶, at a rate of 180 pecks per minute⁷.

We simulated water circulation generated by spinning phalaropes with a motorized toy submarine attached rigidly to a surface float, with the propeller at the same depth as the feet of the bird. The 10-cm submarine generated a rotational eddy in solid-body rotation within the turning circle and an irrotational eddy outside, identical to that produced by phalaropes⁸. Upwelling began nearly instantaneously in the centre of the circle. The water surface bounded the upwelled water, which was deflected radially in a 2-cm layer. The flow was thus strongly three-dimensional, with comparable vertical and radial velocities and an upwelling depth of ten times the radius.

Phalaropes kick water away at the surface so rapidly that the water surface is depressed and deeper water flows upwards to replace it. The bird thus deflects the free surface, driving an upward momentum jet. This intense upwelling differs from classical oceanographic upwelling, wherein vertical velocity is smaller than horizontal velocity by several orders of magnitude. When phalaropes spin they swim sideways (Fig. 2), moving their centre of rotation and extracting food from new areas.

In 1.5-m-deep aquaria, particles 0.5 m under the submarine were upwelled, but in those shallower than 0.5 m a boundary layer developed on the bottom⁹, with central upwelling. When spinning ceased, the bottom was cleared under the turning circle. This also occurred when phalaropes spun over a dish with dead brine shrimp immersed in brine stained with dye. A green, tornado-like tube of dye and prey rose upward from the dish (Fig. 1), rotating opposite to the rotation of the bird as it began to feed.

Spinning concentrates prey into the upwelling jet where the phalarope feeds faster than any other bird. Rapid, localized feeding is effective when food is patchy or out of reach, as with the bird's planktonic prey at sea. Spinning is effective when prey are aggregated and layered against a shallow bottom and subject to accumulation by medially directed bottom currents and when prey are layered or immobilized by cold². Tinbergen noted² that phalaropes do not spin when it is windy. Wind-

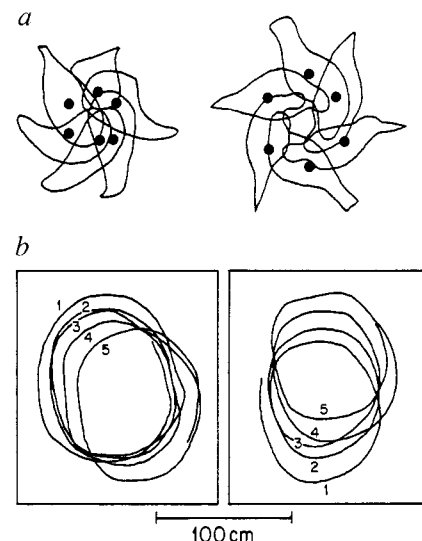


FIG. 2 Motion analysis of a spinning phalarope. Phalaropes in the aquarium were videoed from above and feeding was analysed in two dimensions using an ExpertVision motion analysis system (Motion Analysis Corp., Santa Rosa, California). *a*, Outline of a phalarope during a single spin in a digitized sequence. Filled circles, centroids of the bird's outline in each video frame. *b*, Frame-by-frame centroid tracks for two birds during five sequential spins. Each spin was slightly offset from the former so that the spinning phalarope travelled slowly over the water; spinning velocity for five sequences with three different birds averaged 0.28 m s^{-1} (s.d. = 0.03 m s^{-1}).

generated surface shear, Langmuir cells, and breaking waves may interfere with bird-generated upwelling jets.

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FIG. 1 Spinning red-necked phalarope generates an upwelled plume of prey (brine shrimp) and fluorescein dye. Birds were induced to feed in a 1-m² aquarium by placing brine shrimp in an open petri dish on the bottom, in water shallow enough for the birds to reach the food by dipping the bill and head under water. The water level was then raised gradually until the birds spontaneously spun to feed. Flow patterns were revealed by filling the petri dish with hypersaline water mixed with fluorescein dye and prey that remained in the dish until the birds began to spin.

upwelling, but Sutton¹ and others⁴ report phalaropes "twirling in water many fathoms deep", far too deep for toroidal flow. Instead, red-necked phalaropes produce a subsurface meridional flow that concentrates and upwells prey in an upward momentum jet due to differential effort by the legs (Fig. 1). Each 1-second spin requires 7-8 kicks. The lobed toes spread for thrust and fold for recovery. The outer tarsus travels through an angle of between 90° and 110°, the inner only

Electrophoretic mobility of DNA knots

SIR — Elsewhere in this issue¹, we report a study using Metropolis Monte Carlo simulations to show that populations of thermally distorted knotted polymeric chains maintain certain geometrical properties of the ideal representations of the respective knots. Here we show that, in real DNA knots undergoing gel electrophoresis, there is a linear relationship between speeds of migration of different types of DNA knots and the average crossing numbers of their ideal geometrical representations (the concept of average crossing number is explained in ref. 1).

Several classes of enzymes acting on DNA produce DNA knots, the most well-known being topoisomerases and enzymes participating in site-specific recombination²⁻⁶. By finding out the types of DNA knots formed, it should be possible to determine the mechanisms by which these enzymes are involved in the proper functioning of chromosomes. But it is not straightforward to determine which types of DNA knots are formed by different enzymes. It requires a laborious electron-microscopy technique, where knotted DNA molecules are covered with RecA or UvsX protein to distinguish between underlying and overlying segments of knotted molecules^{7,8}.

Studies combining gel separation of different knot types with electron-

microscope observations have shown that the speed of migration of DNA knots increases with their increasing complexity. To a first approximation, knots with the same minimal crossing number co-migrate on gels⁹. Higher-resolution gels can be used to separate torus- and twist-type knots having the same minimal numbers of crossings¹⁰, opening up the possibility of identifying a given type of knot simply by its position on the gel, without the need for the laborious processes mentioned above^{7,11}.

We therefore decided to analyse the high-resolution gels presented in ref. 10 to compare the migration distances of the knots. When we plotted these distances against the average crossing numbers of ideal representations of the corresponding types of knot, we obtained perfect linear correlation (Fig. 1a). To cross-check if knots other than twist and torus type also migrate proportionally to the average crossing number of their ideal geometric representations, we analysed gels where composite knots ('granny' knots) were run together with different prime knots of the same size¹². Figure 1b shows that granny knots also follow the same rule. This linear relation between the speed of gel migration of the knots and their average crossing number has not previously been noticed, although it has been reported that knots with 'higher' energies migrate quicker than knots with 'smaller' energies¹³.

Obviously, DNA knots cannot maintain their ideal geometrical forms in solution and especially during gel separation. But we have demonstrated that the mean values of the average crossing number calculated for a Boltzmann ensemble of thermally agitated molecules are linearly related to the mean crossing numbers of the ideal geometrical forms of these knots¹. Thus the linear relations in Fig. 1 would be maintained if we used the mean crossing number calculated for the population of thermally agitated configurations of the analysed knotted molecules instead of the average crossing number of ideal forms.

The linear relation between average crossing numbers of knots and their speed of migration can be explained by the fact that the average crossing number is directly proportional to the compactness of knots as expressed by the mean of inverse distances within the analysed trajectory (Fig. 2). The mean of inverse distances in a molecule is an accepted measure of molecular compactness and is simply related to the sedimentation constant¹⁴. Although electrophoretic migration in gels is a more complex process than sedimentation, compact molecules migrate quicker than less compact ones

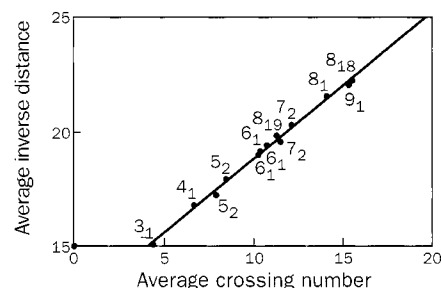


FIG. 2 Compactness of knots as a function of their average crossing number. Mean of inverse distances calculated for several ideal representations of knots is plotted against the average crossing number of these representations of knots. The values of the mean of inverse distances are normalized to the total axial length of a given knot.

with the same charge. Thus it may be not so surprising that different knotted molecules migrate on gels with a speed proportional to their average crossing number.

Because there is no consensus about theoretical models that would allow gel migration of a given type of DNA knot to be predicted, our observation should be helpful to the study of DNA topology. Nevertheless, other gel systems may not preserve the perfect linear relation between migration speed and crossing number of different knots of the same size.

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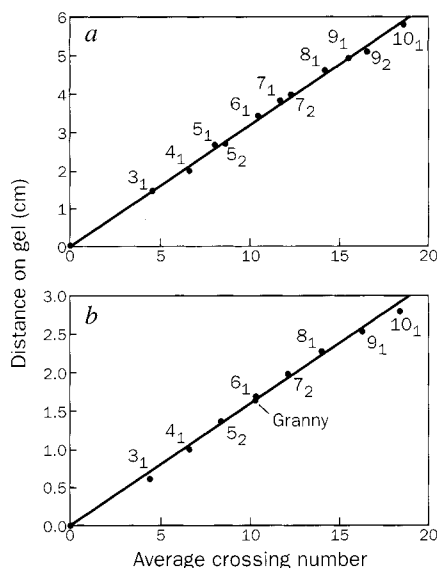


FIG. 1 Linear relation between electrophoretic migration of real DNA knots and the average crossing number of the ideal forms of these knots. a, Distances of different knots from the position of circular DNA measured from the image of a gel shown in Fig. 7 of ref. 10. Note that the knots analysed in ref. 10 were of twist and torus type only. b, Distance of migration of different knots measured on a gel (Fig. 4 of ref. 12). Note that the gel systems used in refs 10 and 12 are very similar.

Origin of patterning in neural tubes

SIR — A notochord and dorsal tubular nerve cord are essential features of chordates, and must have evolved in the most ancient members of our phylum. How complex was the primitive chordate neural tube? Ascidians, as members of the urochordate subphylum, derived from the most basal extant lineage within Chordata¹; anatomically, the nerve cord of ascidian tadpole larvae is simpler than that of vertebrates or cephalochordates, with less than 100 rostral neurons and just axons and ependymal cells in the tail². It is unclear whether this reflects a simple ancestral pattern, or whether it masks hidden developmental complexity.

Here we report the expression of an ascidian (*Halocynthia roretzi*) *Pax* gene,

HrPax-37, descendant from the precursor of *Pax-3* and *Pax-7* in vertebrates. The last two are expressed dorsally in both neural tube and somitic mesoderm; in addition, mouse *Pax-3* mutations reveal a function in differentiation of the dorsal neural tube³⁻⁵. Expression of *HrPax-37* is first detected at the early gastrula stage (*a* in the figure) in the nuclei of six bilateral pairs of cells, destined to form the dorsal part of the neural tube (a8.25, b8.19 and b8.17; the last two also yield muscle cells) or dorsal epidermis (a8.26, b8.20 and b8.18)^{6,7}. By the mid-gastrula stage, expression is maintained only in the first three cells (*b* in the figure). In contrast, at the later neural-plate stage the expression becomes downregulated in the primordial dorsal nerve cells and reactivated in the dorsal epidermis (*c* in the figure). At the neurula stage, just a single row of epidermal cells flanking the presumptive neural tube continues to express the gene (*d* in the figure).

Expression during these early embryonic stages indicates that *HrPax-37* might have a similar function to vertebrate *Pax-3* and *Pax-7* in the differentiation of dorsal neural tube, although *HrPax-37* is also expressed in dorsal epidermis. Epidermal sensory neurons of ascidian larvae are derived from the dorsal epidermal cells^{7,8}, raising the possibility that the expression in this region is comparable with *Pax-3* in vertebrate neural crest³.

During closure of the neural tube, expression fades from dorsal epidermal cells, but is reactivated in the three populations of cells in the neural tube (*e, f* in the figure). By the tadpole larva stage, expression in the most rostral cell population disappears, although extensive expression persists in the sensory vesicle and visceral ganglion (*g* in the figure). At this stage, a striking pattern of *HrPax-37* expression emerges more posteriorly, in the nerve cord of the tail. Expression is detected in roughly 15 reiterated spots, within a dorsal row of cells in the nerve cord (*h* in the figure). To our knowledge,

no segmental structures have previously been reported in the ascidian neural tube. Because this expression must derive from ependymal cells (there are no neuronal cell bodies in the nerve cord²), we suggest this reiterated distribution of *HrPax-37* is an evolutionary remnant reflecting ancestry from a more elaborate segmental organization. This suggestion is compatible with the existence of a segmental ancestor for all urochordates, and would resolve the paradox that appendicularians (also urochordates) have motor neurons segmentally distributed in the tail neural tube^{9,10}.

In conclusion, we suggest that dorsal specification by genes of the *Pax-3/7* subfamily, and segmental organization of neural tube, were established in the ancestors of extant chordates during emergence of the dorsal tubular nervous system.

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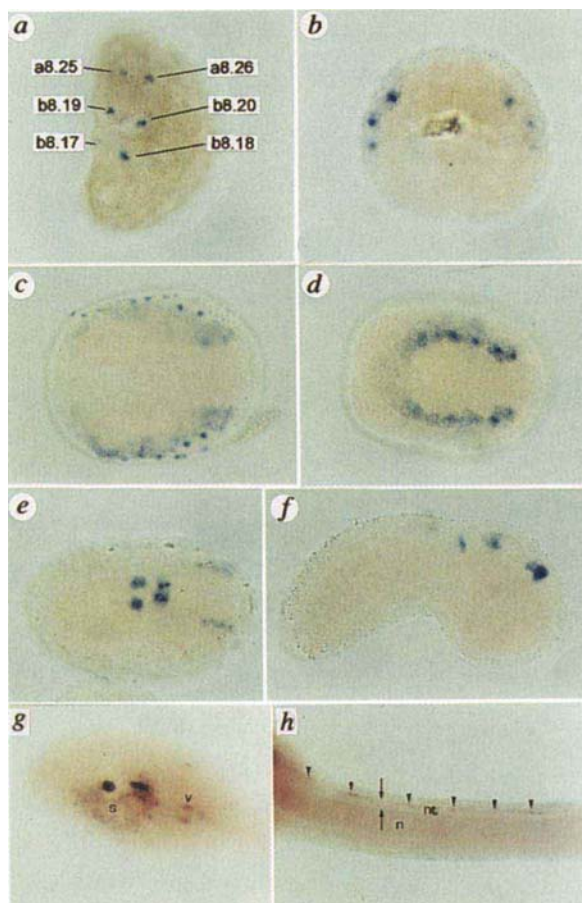
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Neuronal cell death and tPA

SIR — The serine protease tissue plasminogen activator (tPA) has been implicated in brain hippocampal function, as its messenger RNA is present¹ and is rapidly induced in the rat and mouse hippocampus on pharmacological or electrical induction of neuronal activity^{2,3}. In addition, mice deficient in tPA (*tPA*^{-/-})⁴ are resistant to neuronal destruction⁵ after intra-hippocampal injection of high concentrations of glutamate analogues (excitotoxins^{6,7}). Here we investigate whether *tPA*^{-/-} mice are resistant to neuronal degeneration because of pre- and/or postnatal development in the absence of tPA, or whether tPA is necessary for neuronal death in the adult mouse.

An adult requirement for tPA would be significant for two reasons. First, tPA has recently been approved for treatment



Spatial and temporal expression of *HrPax-37* as deduced from *in situ* hybridization. *a*, Lateral view of embryo at early gastrula stage. The signal of b8.17 is out of the plate of focus. *b*, Dorsal view of mid-gastrula embryo. Anterior is to the top in *a* and *b*. *c, d*, Dorsal view of embryo at neural-plate stage (*c*) and neurula stage (*d*). *e*, Dorsal view of the embryo at initiating tailbud stage. The neural tube is closing from the posterior region; in more anterior regions, the expression of *HrPax-37* remains in epidermis. *f*, Lateral view of the embryo at mid-tailbud stage. Anterior is to the right in *c–f*. *g*, Dorsal view of the trunk region of larva. s: sensory vesicle, v: visceral ganglion. *h*, Lateral view of larval tail. The segmental expression of *HrPax-37* in the nerve cord is indicated by arrowheads. Arrows, dorsal and ventral boundary of the neural tube; n, notochord; nt, neural tube. Anterior is to the left in *g* and *h*.

of thrombotic stroke. Because the blood-brain barrier is compromised during stroke, tPA might gain access to the brain parenchyma, thus contributing to neuronal cell death. Second, inhibition of tPA activity in the adult mouse could protect against certain neuronal pathologies.

To distinguish between a developmental or an acute need for tPA, we delivered tPA protein bilaterally to the hippocampus of tPA^{-/-} mice, then injected kainate (a glutamate analogue) on one side, and assessed the extent of neuronal

death. We found that tPA^{-/-} mice infused with buffer (control) did not exhibit significant neuronal degeneration after kainate injection (Fig. 1, top)⁵. In contrast, tPA^{-/-} mice infused with tPA exhibited dramatic neurodegeneration in the kainate-injected hippocampus (Fig. 1, bottom). We detected proteolytic activity¹ equally over both sides of the hippocampus of the tPA-infused tPA^{-/-} mice (verifying that tPA was delivered appropriately), whereas we observed no tPA activity in the control tPA^{-/-} mice. Because we observed degeneration only in the kainate-injected side, tPA alone at this concentration is not sufficient to kill neurons. These results indicate that tPA is required for neuronal death in the adult animal at the time of excitotoxic insult.

As tPA is required to promote neuronal degeneration, we tested whether inhibition of its enzymatic activity might retard neuronal death. We infused mice with plasminogen activator inhibitor-1 (PAI-1; a serine-protease inhibitor that inhibits tPA⁸), injected them with kainate, and assessed neuronal survival in the hippocampus. Wild-type mice infused with buffer were sensitive to neuronal degeneration in the hippocampus (Fig. 2, top). In contrast, mice infused with PAI-1 were resistant to kainate-induced neuronal death (Fig. 2, bottom). The resistance of wild-type mice infused with PAI-1 was comparable to that observed with buffer-infused tPA^{-/-} mice.

Various neuropathological conditions may involve excitotoxic damage to the brain⁹. The identification of extracellular proteases as contributing to the degeneration pathway provides an attractive target for therapeutic intervention. One concern of such therapy in humans would be that inhibition of tPA might lead to clotting disorders. However, tPA^{-/-} mice do not exhibit spontaneous thromboses⁴, indicating that blood homeostasis is possible in the absence of tPA. Therefore, it seems reasonable to investigate further the

effectiveness of protease inhibitors for the therapy of excitotoxic-mediated brain disorders.

Thrombotic stroke is thought to involve an excitotoxic pathway⁹, and tPA can promote neuronal death after excitotoxic insult. Recent studies report treatment of ischaemic stroke within three hours of onset with intravenous tPA^{10,11}. Certainly the excitotoxic cell death caused by injections of kainate does not exactly mimic the damage observed in cerebral ischaemia. Nevertheless, given the possibility that administration of tPA to stroke patients might lead to increased protease levels in the brain parenchyma¹², it seems prudent to examine the potential risk that tPA may cause neuronal death in the already vulnerable tissue.

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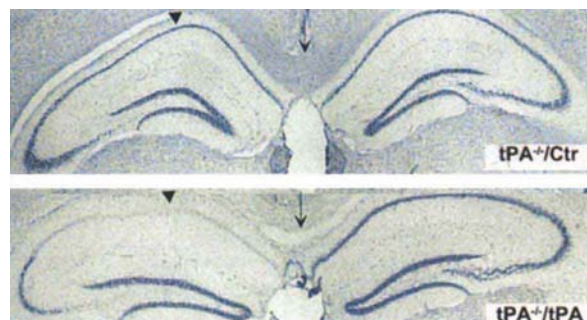


FIG. 1 Intra-hippocampal delivery of tPA restores susceptibility of hippocampal neurons to kainate injury in tPA^{-/-} mice. Coronal sections through the hippocampus of tPA^{-/-} mice stained with cresyl violet to reveal the status of neuronal cells. Mice were infused with PBS (top) or recombinant human tPA (bottom) for 2 days, injected unilaterally with kainate, and the infusion continued for 5 days further, at which point the mice were analysed. Percentage lengths of hippocampal subfields remaining intact after kainate infusion: 100% CA1, 91.2 ± 0.1% CA2/CA3 (top); 10.3 ± 4.4% CA1, 9.1 ± 5.2% CA2/CA3 (bottom). Arrowheads, site of injection; arrows, site of infusion. A micro-osmotic pump (Alzet Inc.) containing buffer (for control animals), or 100 µl human tPA (rtPA, 0.12 mg ml⁻¹), was placed subcutaneously in the back of anaesthetized mice, and a brain infusion cannula connected to the pump was positioned at coordinates: bregma -2.5 mm, medial-lateral -0.5 mm and dorsoventral 1.6 mm, for delivery near the midline. The pump was allowed to infuse (0.5 µl h⁻¹) for 2 days. Mice were then injected with kainate and analysed⁴.

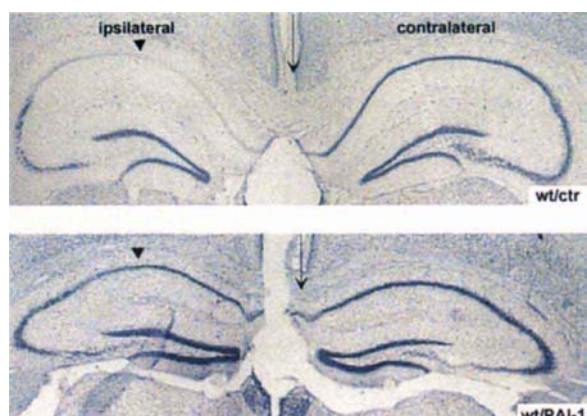
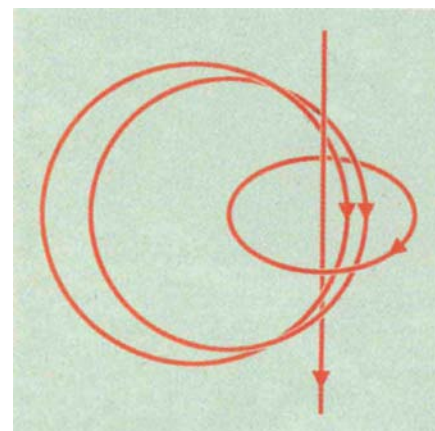


FIG. 2 Plasminogen activator inhibitor type-1 prevents kainate-induced neuronal degeneration. Cresyl violet-stained coronal sections through the hippocampus of wild-type mice. The mice were infused with buffer (top), or mouse PAI-1 (0.12 mg ml⁻¹, American Diagnostica; bottom) for 2 days, and kainate injected as in Fig. 1. Percentage lengths intact after kainate infusion: 6.3 ± 3.1% CA1, 18.1 ± 3.6% CA2/CA3 (top); 68.3 ± 7.4% CA1, 80.9 ± 4.1% CA2/CA3 (bottom). Arrowheads, site of injection; arrows, site of infusion.

Correction



In the Scientific Correspondence "Is ball lightning an electromagnetic knot?" by A. F. Rañada and J. L. Trueba (*Nature* **383**, 32; 1996), Fig. 1, a schematic version of the magnetic lines in the case $n=1$, was printed incorrectly. The correct version is shown above. In Fig. 2a, the origin on the ordinate axis should have read '1'.

The Darwinian family

Howard Gardner

Born to Rebel: Birth Order, Family Dynamics and Creative Lives. By Frank J. Sulloway. Pantheon: 1996. Pp. 653. \$30.

FRANK Sulloway believes that he has identified a primary "engine of historical change". Marx was mistaken in his belief that historical change came about because of clashes between social classes. Freud's claims about the primacy of libidinal urges and Oedipal complexes were similarly mistaken. These influential social scientists should have taken their cue from Sulloway's hero, Charles Darwin. Through pondering the implications of Darwin's principal discoveries, Sulloway has identified a completely unanticipated "engine" of revolution — a "Rosetta stone" in his phrase — the order of one's birth.

In the past, the significance of birth order has intrigued lay people, but those sociologists and psychologists who have studied its effects have come up mainly empty-handed. Sulloway, a historian of science at the Massachusetts Institute of Technology, has altered this situation, probably forever. He has compiled impressive evidence documenting one crucial effect of birth order — although firstborns may be ambitious and achieve a lot in their lifetimes, it is laterborn children who are the "natural rebels" of the book's title.

Described in Darwinian terms, siblings must compete with one another for precious resources, particularly parental attention and support. To secure such support, laterborns differentiate themselves from, and define themselves in opposition to, their elder siblings. And so, with the firstborn typically imitating parental behaviours and attitudes, laterborn youngsters are driven naturally to a posture of opposition — to the embracing of ideas, stances or schemes that are in some sense revolutionary or iconoclastic. Sulloway has reached this conclusion as a result of a thorough review of literally thousands of studies by others, and a painstaking programme of library and questionnaire research extending over 20 years.

Of great interest to Sulloway (and presumably to readers of *Nature*) is the comparison he makes between those who early on supported scientific revolutions (such as the theory of evolution, or Einsteinian relativity) and those of compara-

ble backgrounds and status who opposed these theories. The results of the analyses are staggering. Consider the status of evolutionary ideas before the publication of Darwin's *Origin of Species* in 1859: laterborns were ten times more likely than firstborns to advance evolutionary ideas. By 1875, firstborns had achieved the same level of support for these ideas that laterborns had reached a century before.

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Throughout the debates about evolution at the time, 25-year-old firstborns were as resistant to the theory as 80-year-old laterborns.

Sulloway also provides strong evidence that political revolutions (such as the French revolution) and ideological revolutions (such as Protestantism) were first embraced by the laterborns. Indeed, it is only in the case of revolutions deemed conservative (such as eugenics in science, or counter-revolutions in politics) that supporters are more likely to be drawn from the ranks of the firstborn.

Stated in so bald a form, the theory — and its constituent claims — raises innumerable questions. Bertrand Russell once said that the power of a thinker's contribution lies less in the uniqueness of his ideas, more in the skill with which the thinker defends his views against all possible criticism. Sulloway's approach is admirable in this respect — and particularly so for a work that is primarily historical and archival in nature. Throughout, he considers alternative explanations, produces relevant data where he can, and suggests further studies that could resolve paradoxes and contradictions.

Still, much fancy footwork is required. To begin with, there are questions of definition. Who is really a firstborn, what does it mean to be a functional firstborn — or an "honorary laterborn"? Then there are questions of judgement. Because firstborns so often create theories and make discoveries, Sulloway has to indicate how and why their discoveries differ from those associated with laterborns. When a revolution is supported by a firstborn, the question arises how to decide whether it is conservative or radical.

There are also all the other potential confounding factors, ranging from social class to gender to temperament. Sulloway spends much time explaining "developmental glitches" — for example, why Einstein, Galileo, Newton, Lavoisier and

Bruce Eric Kaplan, *The New Yorker*

Freud all turn out to be firstborns, and yet do not contradict his model. To his credit, Sulloway does not hesitate to tackle such questions. And he declares unequivocally that his whole edifice would be threatened were someone to identify a single, non-conservative revolution that was supported mainly by firstborns.

Born to Rebel contains a treasure-trove of information. Without question, scholars will be examining Sulloway's data (which contain thousands of judgements by other scholars), his multivariate explanatory models, his judgements about birth order and revolutions.

And, without doubt, the story that he tells will be revised to some extent — although I doubt that anyone will ever again claim that the variable of birth order has been shown to be unimportant.

My concerns lie in a different direction. Although I find Sulloway's work fascinating, I am uncertain about where it will lead. One can keep uncovering evidence about the ideological tendencies of laterborns, but Sulloway has already made the case in a knockdown manner. One might find other unknown correlates of birth order, but the history of research in this area gives little reason to expect such remarkable surprises. Freud, Marx and Darwin — the individuals invoked by Sulloway — were distinguished less by being 'right' than by opening entire new worlds of study, by generating bushels of questions that have kept both acolytes and critics busy for generations. Sulloway devotes an appendix to suggestions for future research, but I am uncertain that these will prove as fecund as the present work.

I am a firstborn (at least a functional one — my older brother died just before I was born). So, according to Sulloway's

model, my support of a radical new theory is unlikely. (Only a century from now would someone like me join the Society of the Order of Birth Studies — SOBS). But perhaps I am less of a threat to the theory than laterborns: it is in their tradition — if not their genes — ultimately to carry out the research and initiate the campaigns that will launch the post-Sulloway paradigm.

Clearly, Sulloway intended to write a book “in the grand tradition” and, by and large, he has succeeded in doing so. □

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Of serpents, sharks and shells

Olivia Judson

Sixty Years of Biology: Essays on Evolution and Development. By John Tyler Bonner. Princeton University Press: 1996. Pp. 143. \$24.95, £18.95.

In Search of Nature. By Edward O. Wilson. Island: 1996. Pp. 214. \$19.95, £16.95. Distributed in the United Kingdom by Earthscan.

Privileged Hands: A Scientific Life. By Geerat Vermeij. W. H. Freeman: 1996. Pp. 297. \$23.95, £18.95.

As a boy, John Tyler Bonner spent hours marvelling at the beetles, birds and bones in the Natural History Museum in London.

Edward O. Wilson dreamed of finding a “real serpent”, a snake larger than life — and almost killed himself when he did (it was a massive water moccasin, and it nearly got him when he tried to catch it). Geerat Vermeij collected shells, captivated by their shapes and textures. From early on, each of them knew that nothing in the world was more interesting than biology.

For Vermeij, pursuing this passion has presented a particular difficulty: prejudice. At the age of four, his eyes were removed because of painful and deteriorating glaucoma. As a result of his blindness, teachers doubted that he could study biology; grant agencies doubted that he could do field work; university faculty members doubted that he could teach; and administrators doubted that he could travel safely aboard ships on the high seas. He has conclusively proved all of them wrong.

Yet, perhaps because he is shy or perhaps because he is anxious to show that being blind does not make him different, Vermeij's autobiography, *Privileged Hands*, is patchy. For the most part, it reads as if it is intended to be spoken (with time for questions at the end); he has a tendency to state rather than describe. Too often, his tales of his prolific globe-trotting turn into an annotated thank-you list: many people are mentioned, but few characterized, and the multitude of places he visits rarely come alive in a cacophony of sounds, smells and sensations.

He is at his best when he talks about science — both his own and the work of others. Then, his enthusiasm and curiosity are palpable. His accounts should inspire all weary graduate students. He demonstrates how the pondering of seemingly small questions — for instance, how shells

accumulate the scars and seams that mar their smoothness — can lead to the discovery of large patterns, such as the way that organisms evolve in response to predators.

Of course, one species (us) no longer has to worry about predators. We have exterminated them all. All, that is, but one: *Carcharodon carcharias*, the great white shark. Wilson devotes several pages to the glories of the beast in one of the 12 essays in his delightful new collection. All the essays are reprinted, with some revisions, from book chapters and magazine articles. Wilson even recounts what it is like to be attacked by a shark (although this time it was not personal experience).

Once, aeons ago, predators were a more general problem for us. In several of his essays, Wilson argues convincingly that predators have shaped our minds in profound and lasting ways. Take snakes. According to Wilson, snakes feature in the mythology of every human culture, and many people are petrified of them, even of harmless ones. Why? Perhaps because, for our distant ancestors, survival depended on avoiding potentially dangerous animals. He presents some surprising evidence in support of this idea. Macaques and chimpanzees raised in captivity adopt characteristic fear responses when presented with their first snake. But lemurs, which have spent most of their evolutionary history on a snake-free island, do not show fear.

Unfortunately, although other organisms may have had a deep and lasting influence on the human psyche, humans have had a far more destructive influence on other organisms. The current mass extinction preoccupies Wilson in many of his essays; and he illustrates beautifully how much more dependent we are on other creatures (especially plants and invertebrates) than they are on us. But, best of all, he is able to dazzle and amaze, pretending sometimes to be a termite, sometimes to be a subordinate monkey, and to convey the magical diversity of nature.

Binding that diversity together is one of the trickiest jobs in biology. Bonner is undaunted by the task, however, and in his slim volume of essays he blends ideas that have been on his mind for a while — such as the interaction between natural selection and the physics of organisms — with new ones, such as the way that genes might accumulate and lose functions in the evolution of development or behaviour. One of his recurring themes is the extent to which neutral changes may affect eventual outcomes — using examples from the shapes of the spikes on protozoans to the evolution of ageing.

Like Wilson, and to a lesser extent Vermeij, Bonner likes to contemplate what biology can reveal about humans and human societies. Often comparing human societies with those of slime moulds and ants, he expounds the importance of competition (according to Bonner, computers



LARRY P. TACKETT of the United States was a runner-up in this year's British Gas Wildlife Photographer of the Year Competition with this image of a sea slug laying an egg ribbon. Some of the most stunning entries have now been published in *Wildlife Photographer of the Year: Portfolio Six* (Fountain Press, £24.95).

have become extensions of our drive to compete with each other), and he speculates on the forces that lead human societies to subdivide labour. Although some biologists may find his ideas irritating (can complex behaviours really become innate all of a sudden?), everyone, biologists or not, should find them provocative.

Perhaps these three books — showing in their several ways the importance of studying whole organisms and ecosystems — may help to draw in more research money for ecologists and naturalists. As budgets shrink, these areas are likely — but wrongly — to be among the first to be squeezed. The head of the US National Science Foundation fears that its budget will shrink by 30 per cent in the next five years.

Long gone are the days when, as Bonner reminisces, a letter to the foundation that said “I tried this, that and the other, and nothing really worked” was greeted with the reply: “Don’t worry about it — that is the way research goes sometimes. Maybe next year you will have better luck”. □

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Object lessons in vision

Anya Hurlbert

High-level Vision: Object Recognition and Visual Cognition. By Shimon Ullman. MIT Press: 1996. Pp. 412. £33.95, \$40.

LEGEND has it that about 30 years ago, at the Massachusetts Institute of Technology, the artificial-intelligence pioneer Marvin Minsky instructed a graduate student to connect a camera to a computer and make the computer describe what it saw. What was meant to be a summer project exploded into a huge continuing endeavour, and great advances have since been made in computer vision systems.

But there is still no computer — however well endowed with visual sensors, moving parts and clever algorithms — that can enter an unfamiliar room, immediately process the scene and respond: “Hmm, I’d like to join that little boy playing with the plastic yellow truck, but I’d better not stumble over this pile of puzzles and blocks... hey, is that a red squirrel cavorting on the lawn?”

As Shimon Ullman emphasizes, humans could easily perform the tasks of visual recognition required, even with no previous experience of the scene. Having seen only large grey squirrels before, they could apprehend the small furry red ani-

mal as a member of the same family and know that the squirrel was outside, viewed through a transparent surface. Never having met the child, they could identify its gender and approximate age. And, in the general clutter, they could distinguish individual toys, even where parts of one were obscured by another. Our visual ability to recognize objects is astonishingly flexible and robust.

To recognize an object, the human brain must in some way compare the eye’s image of it with a stored neural representation — to “retrieve information associated with an object, or a class of objects, that is not apparent in the image itself”. But one object may give rise to a multitude of different images, depending on the viewing angle, light-source position, and scene content — and there is not enough room in the brain for all of them.

The problem that the brain has solved is how to match one of the multitudinous incoming images with one of relatively few stored models. Ullman’s book graphically demonstrates just how difficult the problem is and explores possible solutions the brain might have adopted.

The book is not a review or textbook; it is more an idiosyncratic, personal history of Ullman’s own thoughts and contributions to the field. But these contributions are so varied and important, and conveyed with such crystalline logic and precision, that the global overview is inescapable. Half of the book is devoted to the problem of how to recognize an unvarying object from changing viewpoints. In championing the ‘image alignment’ solution, Ullman brings high-level vision down to where it can be explained by straightforward image manipulations that the brain could implement quickly.

One of Ullman’s seminal contributions has been to prove that a three-dimensional model of an object and its viewpoint-varying image may be aligned by matching just three labelled points on each. Beyond that, Ullman shows that it is enough to store a small collection of two-dimensional views of the object, and then to recognize a new incoming view by aligning it with a weighted sum of the stored views (the ‘multiple views’ solution).

These are solutions in the machine-vision tradition, mathematical in conception, computer-oriented in implementation. But brains are biological organs, not machines. Ullman’s computational approach to object recognition was nurtured at the Massachusetts Institute of Technology but has remained distinctly individual because he steers so close to the behavioural and physiological evidence on how the brain works.

He argues convincingly from experimental results that the brain probably uses the multiple-views solution. He presents a biologically plausible model of

how the brain might streamline recognition by operating top-down and bottom-up matching sequences in parallel. He fixes on the most remarkable features of human vision and seeks to explain them.

Why is it that infants learn first how to recognize objects at the class level (horse versus dog, man versus tree) and that in brain-damaged adults the ability to identify individual faces is lost more easily than the ability to recognize faces in general? Fascinatingly, in machine vision it is the other way around: basic-level classification is a more difficult problem than individual identification.

In one of Ullman’s own experiments, he shows that our brains must employ special class-based recognition routines for human faces: we are intrinsically poor at recognizing upside-down faces, although we can improve with training. But we remain very bad at recognizing upside-down faces under varying illumination, whereas we can easily recognize upright faces under any shadow. So there must be automatic mechanisms for discounting shading variations, specific to upright faces.

The book is illustrated throughout with black-and-white line drawings of objects ranging from Saabs to roosters. The primal impact of these simple contours emphasizes how powerful our vision is, and how much machines still have to learn. □

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Mum’s a test-tube

Benno Müller-Hill

Quest for Perfection: The Drive to Breed Better Human Beings. By Gina Maranto. Scribner: 1996. Pp. 335. \$25.

THE prize-winning science journalist Gina Maranto’s book about the history, present state and possible future of test-tube fertilization begins light-heartedly. Jamie Grifo, a reproductive endocrinologist at New York University, is “the kind of doctor nurses adore”. Grifo has a “repertoire of quips and jokes (some of them slightly ribald) which he deploys throughout the day (What does President Clinton say to his wife after sex?... Hi, honey, I’ll be home in 20 minutes)”.

The reader who expects more amusement of this sort will be disappointed. In the first half of the book, Maranto writes about the history of her subject. What did people do with their babies during the Palaeolithic period? What did the

ancient Greeks do? We learn a lot about ancient infanticide. We learn that "Sparta was a community that took sex seriously". And then, of course, we learn about the eugenic ideas of Plato. We read about the Romans and the Hebrews, and all this is interesting. The reader may wonder why the Minotaurus is not worth mentioning. Daedalus, after all, was also active as a gynaecologist.

We are introduced to the Middle Ages in a chapter called "Sanctity, body heat, bad cess, and blood". From there we go on to the Renaissance in a chapter entitled "Of eggs and sperm", which begins with the sentence: "By the time the master frescoist Giotto began working on the elegant yet emotionally charged cycle of paintings whose brilliant hues cover every square inch of the barrel-vaulted Madonna dell Arena Chapel in Padua, then an independent republic neighbouring Venice, the university there was already a century old, having opened its doors in 1222." Too much, too much! We also learn about Harvey, Malpighi, Swammerdam, Leeuwenhoek, von Baer and Weismann. The chapter ends with a brief mention of "an obscure monk

named Gregor Mendel". So much for the first 100 pages. The next 50 deal with various forms of eugenics including Nazi racial hygiene. Here the writing becomes concise.

The real message of the book begins in the second half. It is worthwhile to read what the author has to report here about artificial insemination of animals and women. She tells us that "during the 12 months spanning 1986 and 1987, there were some 172,000 artificial inseminations in the United States of which about half were with donor sperm". The author points out the paradox that the veil of secrecy over the procedure makes an objective evaluation impossible.

Here too is the fascinating story of the slow path to success with human *in vitro* fertilization trodden by Edwards and Steptoe in 1978. Today's success rate with the technique is still not much higher than 20 per cent. The "average price tag for an IVF baby (as of 1989) was US\$50,000". Of course, little of this is paid by medical insurance — surrogate mothers get \$2,000. It is big business now. The freezers are full of pre-embryos and have to be cleaned out from time to time. When that happened

in the United Kingdom a couple of months ago, it caused an outcry.

Maranto also writes about preimplantation diagnosis. A single cell may be removed from a cleaving embryo without damaging the remaining embryo. This isolated cell may be analysed by the polymerase chain reaction or fluorescence *in situ* hybridization for the presence or absence of a certain genotype. To do so is high technology and not ordinary medicine. Worldwide, some 30 such children have been born. The author cites one case where the test for the presence of a Y chromosome went wrong. The embryo was later aborted.

These last chapters provide the real interest of the book. For those who want to know more, the author has prepared an impressive list of references, all in English. Finally, the last sentence is a memento for all involved, however borderline, in such work: "Where love, compassion, altruism, and justice have failed, genetic manipulation will not succeed." □

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Textbooks at a glance

Excellent ★★★★★ Good ★★★★ Fair ★★★ Poor ★

Stars as Laboratories for Fundamental Physics

By Georg G. Raffelt
University of Chicago Press
\$77, £61.50

THE Standard Model of particle physics has been spectacularly successful. Yet there are hints of new physics (particularly involving neutrinos), and questions remain unanswered. Particle physicists are increasingly turning to astrophysics as they search for clues to lead them beyond the Standard Model — clues, for example, about neutrinos with finite mass or non-standard interactions, or the existence of axions or other new particles. The burst of neutrinos seen from supernova 1987A was well explained by models of the supernova collapse process. A flux of undetected axions would have produced an extra channel for energy loss from the collapsing core, so that the observed neutrino emission in fact set stringent limits on the presence of axions.

Georg Raffelt gives an extensive and expert review of this new speculative research area on the boundary between particle physics and astrophysics. He emphasizes the fundamental physics, and provides a detailed compendium of results for the serious student.

Michael L. Cherry Department of Physics and Astronomy, Louisiana State University, USA

Range	★★★★
Depth	★★★★
Accuracy	★★★★
Up-to-dateness	★★★★
Accessibility	★★
Style	★★

Foundations of Geomagnetism

By George Backus, Robert L. Parker & Catherine Constable.
Cambridge University Press
£40, \$59.95

THE invention of the compass and its use for navigation propelled the study of the Earth's magnetic field, geomagnetism, to the forefront of natural science. It remains an exciting research area.

George Backus's lecture notes, developed over many years, are well known among theoretical geophysicists as an authoritative source for the subject's mathematical foundations. The lectures on geomagnetism have now been edited for publication.

The result is no ordinary textbook. It concentrates on rigorous mathematical development; although the main field, palaeomagnetism, and the external fields are treated well, no attempt is made to be comprehensive or up to date. For example, electrodynamics and mathematics applicable to spherical geometry occupy over half the book, whereas mean-field theory and dynamics of dynamos get six pages each.

This is essential reading for any mathematics graduate working in geomagnetism and useful to others working in spherical geometry; it will remain authoritative for many years to come.

David Gubbins Department of Earth Sciences, University of Leeds, UK

Range	★★★★
Depth	★★★★
Accuracy	★★★★
Up-to-dateness	★★
Accessibility	★
Style	★★★★

Understanding Fossils: An Introduction to Invertebrate Palaeontology

By Peter Doyle
Wiley
£17.99, \$34.50 (pbk)

A LARGE difficulty faced by those who teach palaeontology is striking a balance in undergraduate courses between source data — the systematics and ranges of the fossil groups — and their application.

This new book presents one man's solution, condensing into a single volume straightforward descriptions of the main taxonomic groups from foraminifera to trilobites, as well as trace fossils, together with case histories showing their applications in studies of evolution, ancient environments and stratigraphy.

The sheer concentration of topics occasionally reduces the text to almost a list. Nonetheless, this is a user-friendly treatment that will make a good course text supplemented with the latest papers from the literature. But there remains scope for a book that weaves data from fossil invertebrates, as presented here, into an account of vertebrates (including dinosaurs) and plants, allowing the student to appreciate the importance of the fossil record as a whole.

Derek E. G. Briggs Department of Geology, University of Bristol, UK

Range	★★★★
Depth	★★★★
Accuracy	★★★★
Up-to-dateness	★★★★
Accessibility	★★★★
Style	★★

The tumour-suppressor gene *patched* encodes a candidate receptor for Sonic hedgehog

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The protein Sonic hedgehog (Shh) controls patterning and growth during vertebrate development. Here we demonstrate that it binds Patched (vPtc), which has been identified as a tumour-suppressor protein in basal cell carcinoma, with high affinity. We show that Ptc can form a physical complex with a newly cloned vertebrate homologue of the *Drosophila* protein Smoothed (vSmo), and that vSmo is coexpressed with vPtc in many tissues but does not bind Shh directly. These findings, combined with available genetic evidence from *Drosophila*, support the hypothesis that Ptc is a receptor for Shh, and that vSmo could be a signalling component that is linked to Ptc.

THE vertebrate homologues of the *Drosophila* segment polarity gene *hedgehog* (hh) compose a family of at least five proteins^{1–6}. The most notable of these is Sonic hedgehog (Shh), which is expressed in multiple embryonic tissues including the ventral forebrain, notochord, floorplate, limb bud and hindgut endoderm. Shh seems to be involved in the specification of multiple ventral cell types including motor^{7,8}, serotonergic⁹ (M.H. and A.R., unpublished results), dopaminergic¹⁰ and forebrain¹¹ neurons in the neural tube, and in the formation of sclerotome, vertebra and ribs^{12,13}. In the embryonic limb bud, Shh is likely to be the signal required for anterior posterior patterning³ and for the establishment of a functional apical ectodermal ridge^{14,15}, whereas in the hindgut Shh produced by the endoderm has been implicated in the specification of visceral hindgut mesoderm¹⁶. The critical role of Shh in tissue patterning is further illustrated by the finding that mice deficient in Shh die before birth and display multiple developmental defects including cyclopia, absence of ventral cells in the neural tube, absence of the spinal column and most of the ribs, absence of distal limb structures, and degeneration of the notochord¹⁷.

Other members of the Hedgehog protein family have also been implicated in tissue patterning. For example, Desert hedgehog (Dhh) was shown to be essential for testes development¹⁸, Indian hedgehog (Ihh) is required for chondrocyte differentiation¹⁹, and zebra-fish-specific *Echidna* and *Tiggy-winkle* hedgehog are involved in muscle-cell specification³ and eye patterning⁶, respectively.

Despite the importance of Hedgehog proteins in vertebrate development, little is known about their receptors or mechanism of action. Genetic evidence in *Drosophila* implicated two proteins as candidate receptors for Hh: Patched (Ptc)^{20–23} a putative 12 transmembrane (TM) protein; and Smoothed (Smo)^{24–27}, a

7 TM protein. Ptc is thought to be a negative regulator of Hh, and the Hh signalling cascade seems to be constitutively active in its absence. In contrast, Smo is an essential component in the Hh pathway, and Smo mutants display the same phenotype as Hh mutants.

Recently, vertebrate homologues of Ptc (but not of Smo) have been identified in the chick²⁸, mouse²⁹ and human^{30,31}. Furthermore, the human homologue of Ptc (hPtc) was found to be mutated or inactivated in the basal cell naevus syndrome (BCNS), a familial complex of cancers and developmental abnormalities, and in basal cell carcinoma (BCC), leading to the proposal that hPtc is a tumour-suppressor gene^{30,31}.

Given the physiological and clinical importance of the vertebrate Hh, we attempted to identify receptor candidates for these molecules.

Isolation of a vertebrate Smo homologue

Studies in *Drosophila* suggested that Smo is a receptor for Hh^{26,27}, so we began by searching for vertebrate homologues of Smo. A complementary DNA library, generated from Shh-responsive, embryonic day (E) 9–10 rat tissues, was screened at low stringency with a *Drosophila* Smo (dSmo) probe, and overlapping cDNAs were isolated which encoded a protein of 794 amino acids. Subsequently, a human Smo homologue, which is 94% identical to the rat Smo, was identified. The human and rat Smo proteins (hSmo and rSmo) are 33% homologous to dSmo (homology in the transmembrane domains is 50%), 23% homologous to the *Drosophila* Wingless receptor³², and 25% homologous to the vertebrate Frizzled protein³³. Like their *Drosophila* homologue^{26,27}, hSmo and rSmo appear to be 7 TM G-protein-coupled receptors³⁴, possessing four glycosylation sites and a putative extracellular amino terminus 203–205 amino acids long, which

includes 13 stereotypically spaced cysteines and could bind a polypeptide ligand (Fig. 1).

Tissue distribution of the vertebrate Smo

Examination of mouse tissues by northern blot analysis using rSmo as a probe demonstrated the presence of an approximately 4.4-kilobase *Smo* transcript from embryonic day-7 onwards (Fig. 2a). *In situ* hybridization analysis further revealed that the rat *smo* messenger RNA was found in Shh-responsive tissues, such as the early neural folds and neural tube^{1,2,4,7,8,10}, pre-somitic mesoderm and somites^{12,13}, and developing limb bud³, gut¹⁶ and eye²⁶. Transcripts for *smo* were also observed in those tissues with development regulated by other members of the vertebrate Hh protein

family, such as embryonic testes (Dhh)¹⁸, cartilage (Ihh)¹⁹ and muscle (the zebrafish *Echidna* Hh)⁵ (Fig. 2b and data not shown). In all of these tissues, the temporal and spatial distribution of rSmo and rPtc shows considerable overlap. For example, in the embryonic nervous system, *smo* and *rptc* are initially expressed throughout the neural folds and early neural tube (*smo* mRNA is evenly distributed along the dorsal-ventral axis, whereas *rptc* mRNA is found at higher levels ventrally) (Fig. 2b, E9, E10). By E12, expression of both *smo* and *rptc* declines dramatically in lateral parts of the neural tube, and by E15 their mRNAs are restricted to cells which are in close proximity to the ventricular zone (Fig. 2b, E12, E15). The *smo* and *rptc* mRNAs are also found adjacent to Shh-expressing cells in the embryonic lung, epiglottis,

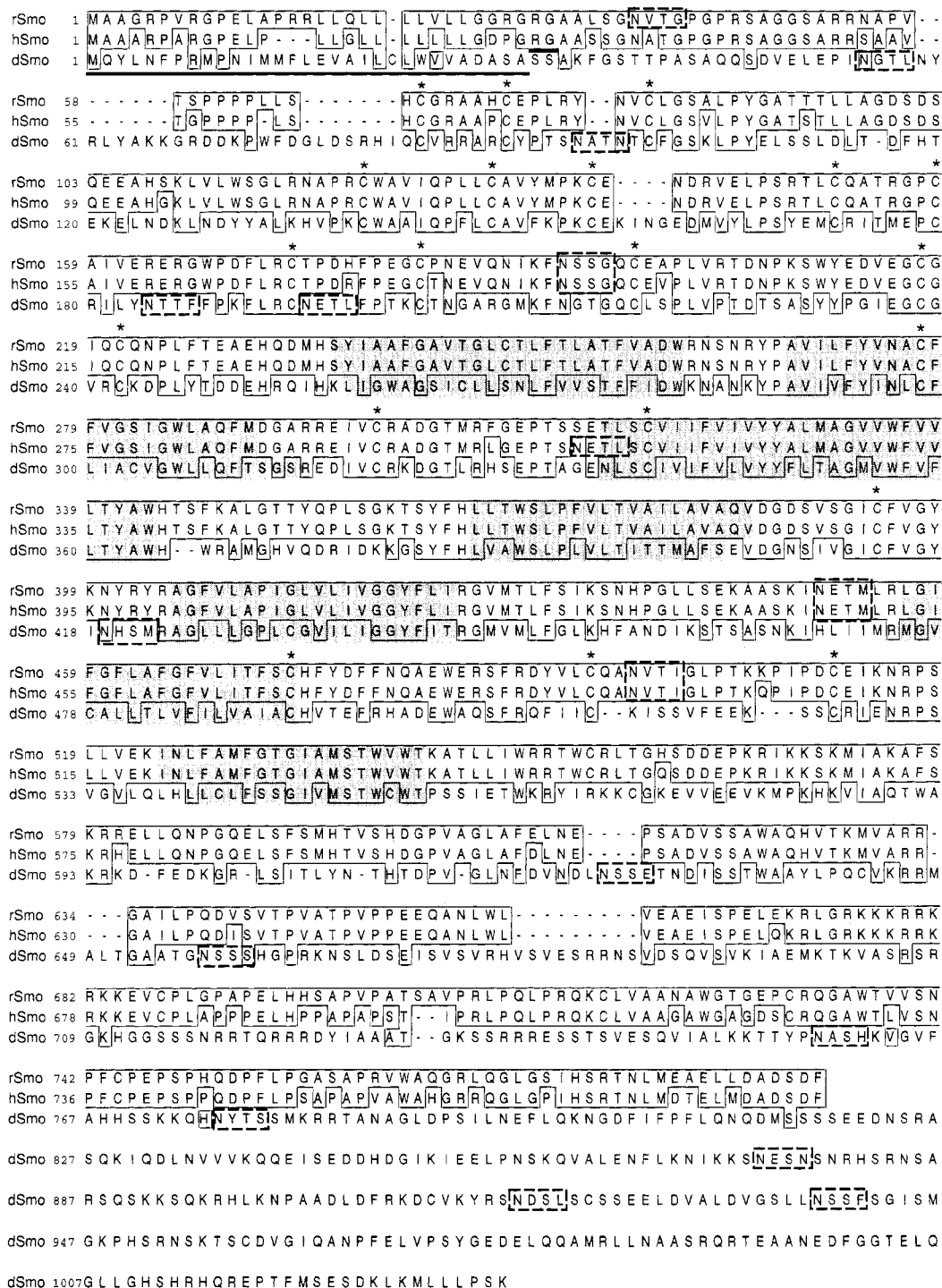


FIG. 1 The primary structure of hSmo and rSmo, and their homology to dSmo. Signal peptide sequences are underlined, conserved amino acids are boxed, cysteines are marked with asterisks, potential glycosylation sites are marked with dashed boxes, and the 7 hydrophobic transmembrane domains are shaded.

thymus, tongue, jaw, taste buds and teeth, as well as in other tissues that do not contain high levels of *Shh* mRNA, such as the skin (Fig. 2b; compare top panels of Shh, Smo and Ptc). In adults, *smo* mRNA was found in multiple tissues including heart, brain, liver, lung, skeletal muscle, kidney and testis (Fig. 2a).

Shh does not bind Smo

To characterize a possible physical association between Shh and rSmo, competition-binding, cross-linking and co-immunoprecipitation experiments were performed. Surprisingly, given the receptor-like structure of rSmo and the genetic evidence that dSmo is a receptor for *Drosophila* Hh^{26,27}, no direct interaction between mouse Shh and rSmo could be detected. Specifically, we did not detect any binding of IgG-Shh-N chimaeric protein (the N-terminal biologically active portion of Shh³⁵ fused in-frame to the Fc portion (the crystallizable fragment) of human IgG- γ 1) (Fig. 3b), or of epitope-tagged Shh or Dhh (data not shown) to

cells that expressed rSmo (Fig. 3a). Similarly, ¹²⁵I-Shh-N could not be co-immunoprecipitated by antibodies against an epitope-tagged rSmo (Fig. 4c), nor could the IgG-Shh-N chimaeric protein immunoprecipitate rSmo (Fig. 4a). Finally, no cross-linking or equilibrium binding between ¹²⁵I-Shh-N and rSmo could be demonstrated (Fig. 4b and data not shown), and no binding of tagged *Drosophila* Hh to rSmo or to dSmo could be detected (data not shown).

Shh binds vPtc with high affinity

Although we cannot rule out the possibility that additional vertebrate homologues of dSmo exist which may bind Shh directly, our findings argue against the hypothesis that rSmo, acting alone, is a receptor for Shh or Dhh. However, the possibility remained that rSmo might be an essential component of a putative Shh receptor complex, of which the ligand-binding function is provided by another protein. We therefore examined whether vPtc, the only other transmembrane protein known to be associated with the Hh signal cascade^{20–23,28,29}, could be a ligand-binding component of such a receptor complex. Indeed, we found that epitope-tagged Shh-N, as well as IgG-Shh-N, bind specifically and saturably to 293 cells expressing the mouse Ptc (mPtc; mPtc is 33% identical to its *Drosophila* counterpart) (Fig. 3c–e and data not shown). (Preliminary experiments suggest that *Drosophila* Hh also binds to mPtc.) Furthermore, mPtc was immunoprecipitated by IgG-Shh-N (Fig. 4a), and antibodies to an epitope-tagged mPtc readily co-immunoprecipitated ¹²⁵I-Shh-N (Fig. 4c). Finally, competition-binding experiments demonstrated that ¹²⁵I-Shh-N could be crosslinked to mPtc (Fig. 4b), and that the two proteins interacted with a K_d of 460 pM (Fig. 4d). No binding of ¹²⁵I-Shh-N to parental 293 cells or to cells expressing exogenous rSmo could be detected (data not shown), and no change in the affinity between Shh-N and Ptc was observed in the presence of transfected rSmo (Fig. 4d). However, given the wide tissue distribution of Smo (Fig. 2), we cannot exclude the possibility that low levels of

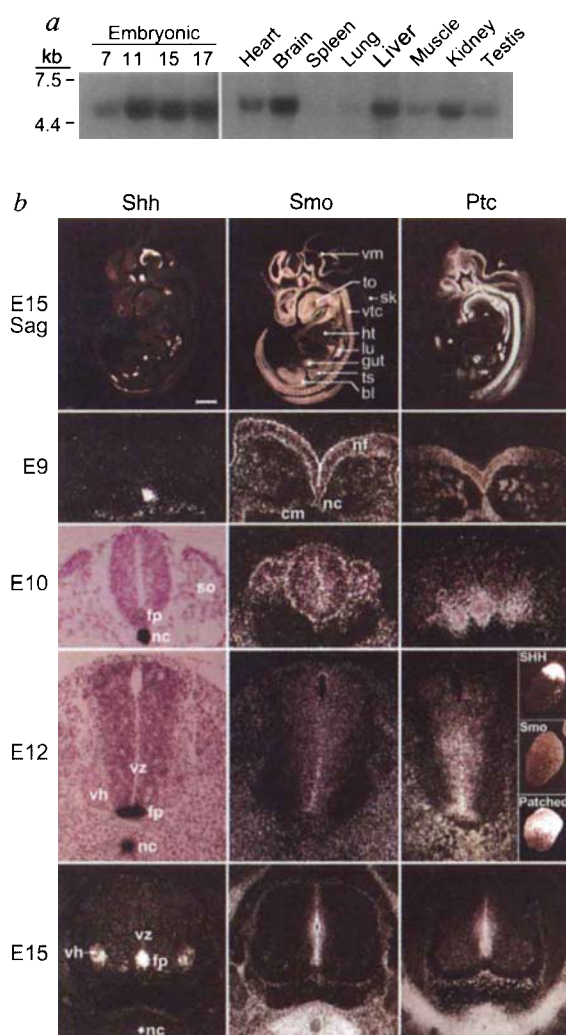


FIG. 2 Tissue distribution of Shh, rSmo and rPtc. a, Northern blot analysis of mouse and rat tissues with rSmo probe. b, *In situ* hybridization of Shh (left), rSmo (middle) and rPtc (right, not including insets) to rat tissues. Row E15 Sag, sagittal sections through E15 rat embryos; rows E9, E10, E12 and E15, coronal sections through E9 neural folds, E10 neural tube and somites, and E12 and E15 neural tube. Insets in row E12 show sections through forelimb bud of E12 rat embryos. Abbreviations: bl, bladder; cm, cardiac mesoderm; fp, floorplate; ht, heart; lu, lung; nc, notochord; nf, neural fold; sk, skin; so, somite; to, tongue; ts, testes; vh, ventral horn; vm, ventral midbrain; vtc, vertebral column; vz, ventricular zone. Scale bar shown in top left panel is 700 μ m in row E15 Sag; 50 μ m in E9; 45 μ m in E10; 40 μ m in E12; 140 μ m in E15; and 720 μ m in insets.

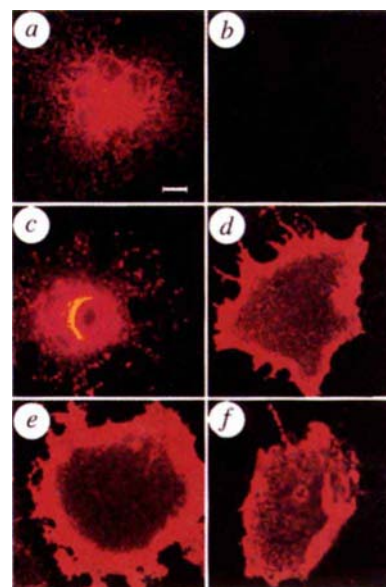


FIG. 3 Shh-N and Dhh-N bind to cells expressing mPtc but not to cells expressing rSmo. Staining of COS-7 cells expressing the Flag-tagged rSmo (a, b) or Myc-tagged mPtc (c–f), with anti-Flag (Smo) antibody (a), anti-Myc (mPtc) antibody (c), IgG-Shh-N (b, d), a Flag-tagged Shh-N (e), a Flag-tagged Dhh-N (f). Only cells expressing mPtc bind the various tagged forms of Shh-N and Dhh-N. No binding of other IgG fusion proteins to mPtc-expressing cells and no binding of the various tagged forms of Shh-N to untransfected cells were detected (data not shown). Scale bar, approximately 5 μ m.

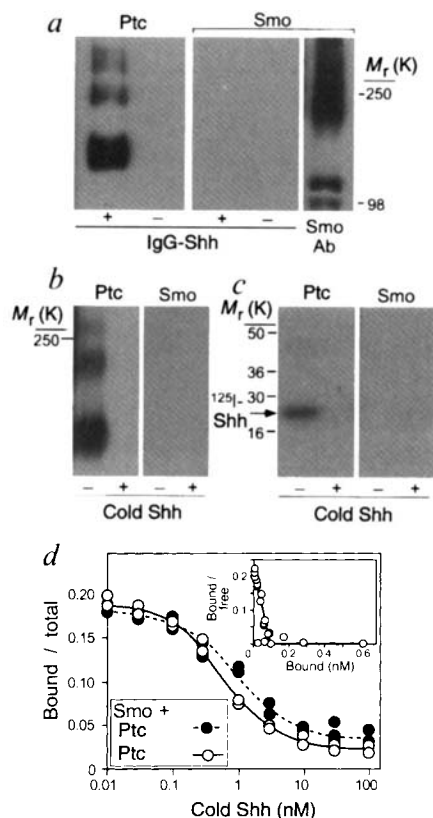


FIG. 4 Shh-N binds to mPtc but not to rSmo. *a*, Co-immunoprecipitation of epitope-tagged mPtc (Ptc) or epitope-tagged rSmo (Smo) with IgG-Shh-N (IgG-Shh), from cells expressing either mPtc or rSmo alone, as indicated. Only mPtc could be co-immunoprecipitated by the IgG-Shh-N protein. Immunoprecipitation of rSmo could be achieved only with antibodies to the Smo epitope tag (Smo Ab). *b*, Cross-linking of 125 I-Shh-N (125 I-Shh) to cells expressing mPtc or rSmo in the absence or presence of excess unlabelled Shh-N (Cold Shh—+/-). 125 I-Shh-N could be cross-linked only to mPtc-expressing cells. *c*, Co-immunoprecipitation of 125 I-Shh-N by antibodies to Myc-tagged mPtc (Ptc) or to Flag-tagged rSmo (Smo). 125 I-Shh-N could be co-immunoprecipitated with antibodies to the Myc-tagged mPtc, but not with antibodies to the Flag-tagged rSmo, and only in cells that expressed mPtc. *d*, Competition binding of purified recombinant 125 I-Shh-N to cells expressing mPtc or mPtc + rSmo. The Scatchard analysis (inset) gave a K_d value of 460 pM. No binding to cells expressing rSmo alone was observed (not shown), and there was no increase in binding affinity to mPtc in the presence of rSmo. Labels above photographs: Ptc, cells transfected with a Myc-tagged mPtc alone; Smo, cells transfected with a Flag-tagged rSmo alone.

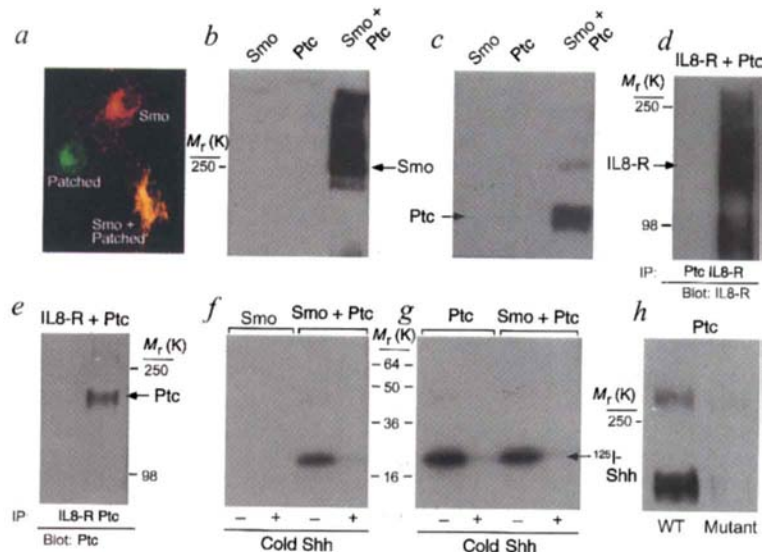
endogenous Smo expressed in 293 cells contributed to the Shh-N-Ptc interaction. In addition, we find that Shh is not the only member of the Hh protein family that binds Ptc: another member of the Hh protein family, Dhh also binds specifically to cells expressing Ptc (Fig. 3f).

Ptc, Smo and Shh form a physical complex

We next determined whether Ptc and Smo are present in a co-immunoprecipitable protein complex. Human embryonic kidney

(293) cells were transiently transfected with expression vectors for epitope-tagged rSmo (Flag epitope) and mPtc (Myc epitope), cells were lysed, and solubilized protein complexes were immunoprecipitated with the antibody to one of the epitopes and then analysed on a western blot using the antibody to the other. In cells expressing mPtc or rSmo alone, no protein complexes were detected. Similarly, in cells that expressed mPtc together with either a gD-tagged transmembrane tyrosine kinase receptor, *trkB*³⁶, a flag-tagged 7 TM receptor for interleukin-8 (ref. 37) or a flag-tagged rat Frizzled³³, no co-immunoprecipitation of Ptc was observed when lysates were immunoprecipitated with antibodies to the various epitope tags (Fig. 5d, e and data not shown). In contrast, in cells expressing both mPtc and rSmo (Fig. 5a), rSmo was readily co-immunoprecipitated by antibodies against the epitope-tagged mPtc (Fig. 5b), and mPtc was readily co-immunoprecipitated by antibodies against the epitope-tagged rSmo (Fig. 5c) (approximately 30% of the immunoprecipitable rSmo and mPtc were found in a complex). Further, 125 I-Shh-N could be co-immunoprecipitated by antibodies against either the epitope-tagged rSmo or mPtc, from cells expressing both rSmo and mPtc, but not from cells expressing rSmo alone (Fig. 5f, g). These

FIG. 5 Ptc and Smo can form a protein complex. *a*, Double immunohistochemical staining of mPtc (green) and rSmo (red) in transfected cells. Yellow indicates coexpression of the two proteins. *b*, *c*, Detection of mPtc-rSmo complex by co-immunoprecipitation from dual-transfected cells. *b*, Immunoprecipitation with antibodies to the Myc-tagged mPtc and analysis on a western blot with antibodies to Flag-tagged rSmo. *c*, Immunoprecipitation with antibodies to the Flag-tagged rSmo and analysis on a western blot with antibodies to Myc-tagged mPtc. Protein complexes containing both rSmo and mPtc are readily detected in both cases. *d*, *e*, Immunoprecipitation with antibodies to the Flag-tagged interleukin-8 receptor (IL8-R) or to Myc-tagged mPtc from cells expressing both flag-tagged IL8-R and Myc-tagged mPtc, and analysis on a western blot with anti-Flag antibody (IL8-R) (*d*) or anti-Myc antibody (mPtc) (*e*). *f*, *g*, Co-immunoprecipitation of 125 I-Shh-N bound to cells expressing rSmo and mPtc or mPtc alone, with antibodies to either rSmo (*f*) or mPtc (*g*) epitope tags. Protein complexes containing both rSmo and 125 I-Shh-N are detected only in cells that express both mPtc and rSmo. *h*, Western blot showing the transient expression level of a Myc-tagged, wild-type (WT) or mutated Ptc protein in 293 cells; the mutant retained a complete open reading frame (insertion of amino acids Pro-Asn-Ile after amino acid 815) (ref. 31). The level of the mutated Ptc protein (Mutant) was less than its wild-type counterpart. Labels above photographs: Ptc, cells transfected with a Myc-tagged mPtc alone; Smo, cells transfected



with a Flag-tagged rSmo alone; Smo + Ptc, cells transfected with expression vectors for both proteins; IL8-R + Ptc, cells transfected with expression vectors for both Flag-tagged IL8-R and Myc-tagged mPtc.

findings are consistent with the idea that Shh-N, rSmo and mPtc are present in the same protein complex, and that a Smo-Shh-N complex does not form in the absence of Ptc.

Discussion

We have provided evidence that mPtc binds Shh-N with high affinity; that mPtc and rSmo are co-expressed in multiple tissues; and that these two proteins can form a complex to which Shh-N binds. We have not demonstrated that rSmo indeed functions in the Shh pathway in a manner similar to its *Drosophila* counterpart^{26,27}, nor have we excluded the possibility that vPtc can transduce signals independent of vSmo, or that Shh-N uses additional receptors. With these limitations in mind, our findings, combined with previous genetic studies in *Drosophila*^{26,27} and human^{30,31}, are best explained by the hypothesis that vPtc is a ligand-binding component, and vSmo a signalling component, in a multi-subunit Shh receptor complex (Fig. 6). In other multi-component receptor systems, the ligand-binding subunits often act as activators^{38,39}, but here, as previously postulated²³ and as our data confirm, the Hh-binding protein (vPtc) appears to be a ligand-regulated suppressor of a signalling unit (vSmo) (Fig. 6). It remains to be determined whether Smo is constitutively active in the absence of Ptc or whether, under these circumstances, Smo would still require a specific ligand for activation.

Genetic mutations leading to a truncated^{30,31} or unstable (Fig. 5h) Ptc protein appear to be associated with familial and sporadic forms of BCC. This correlation, combined with the fact that Ptc is a high-affinity binding protein for Shh, suggests that the Hh system may provide mitogenic or differentiative signals to basal cells in the skin throughout life. Furthermore, our findings raise the possibility that BCNS and BCC might result from constitutive activation of vSmo, which becomes oncogenic after its release from inhibition by Ptc (Fig. 6).

Notably, Shh is not the only Hh protein that interacts directly with Ptc. As we have demonstrated, at least one other member of this protein family (Dhh) can bind Ptc. These results suggest that multiple Hh proteins can mediate their distinct functions through a single receptor system.

With a receptor for Shh (and Dhh) identified, it will now be possible to study the mechanism by which the Hh family exerts mitogenic, differentiative and morphogenic effects at the molecular level. Furthermore, the identification of Smo as a candidate oncogene in familial and sporadic BCCs may lead to a better understanding of, and eventually to a therapy for, this common human cancer. □

Methods

Cloning of the rat Smo homologue. The rat Smo homologue (rSmo) was identified by screening a rat E9–10 cDNA library at low stringency with a probe encompassing the entire coding region of *Drosophila smoothened*²⁶. Eight positive plaques were identified, and three overlapping cDNA clones were

sequenced. cDNAs for the human homologue of Smo (hSmo) were isolated from a human embryonic lung library (Clontech), using the rat cDNA as probe.

Northern blot analysis and *in situ* hybridization. For northern analysis, mouse embryonic and rat multiple tissue northern blots (Clontech) were used. For *in situ* hybridization, E9–15.5 rat embryos were immersion-fixed overnight at 4 °C in 4% paraformaldehyde and cryoprotected overnight in 20% sucrose. P1 rat brains were frozen fresh. All tissues were sectioned at 16 µm, and processed for *in situ* hybridization using ³³P-UTP labelled RNA probes³⁹.

Expression constructs and immunohistochemistry. cDNAs for the various proteins were cloned into a cytomegalovirus-based expression vector, and epitope tags added to the extreme C terminus by PCR-based mutagenesis. For mPtc cDNA, the Myc epitope tag was added after amino acid 1293, with an engineered stop codon immediately following. This resulted in a protein partially truncated at the C terminus. Experiments with the full-length *mptc* demonstrated similar Shh binding (data not shown). The *ptc* mutant was generated by site-directed mutagenesis of the Myc-tagged *mptc* cDNA. For visualization of protein expression, COS-7 cells, transiently transfected with expression constructs, were stained using anti-Flag M2 (IBI) or anti-Myc antibodies permeabilized and (Invitrogen or A-14; Santa Cruz), followed by Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch) and/or biotinylated anti-rabbit IgG (Molecular Probes). For visualization of Shh binding, COS-7 cells transiently expressing rSmo or mPtc were exposed to tagged Shh or Dhh (2 h at 4 °C), fixed, and stained with a Cy3-conjugated anti-human IgG (for IgG-Shh-N) or anti-Flag antibody (for Flag-tagged Shh-N or Dhh-N) followed by Cy-3 conjugated anti-mouse IgG.

Protein production. Human embryonic kidney 293 cells were transiently transfected with expression vectors encoding either IgG-Shh-N (Shh-N fused in frame after amino acid 198 to the Fc portion of human IgG-γ1), flag-tagged Shh-N, or flag-tagged Dhh-N (the flag epitope was added immediately after the internal proteolytic cleavage site) and medium was collected after 48 h. Mouse Shh-N was produced and purified as described¹⁰.

Immunoprecipitation, competitive binding and cross-linking analyses. Embryonic kidney 293 cells transiently transfected with either Flag-tagged rSmo or Myc-tagged mPtc were used in all of the above experiments. For the co-immunoprecipitation, cells were incubated in the presence or absence of the IgG-Shh-N chimera (1 µg ml⁻¹, 30 min at 37 °C), or in the presence of ¹²⁵I-Shh-N with or without an excess of unlabelled Shh-N (2 h at 4 °C) and then lysed³⁸. Lysates were centrifuged and soluble protein complexes were immunoprecipitated with either protein A-Sepharose (for the IgG-Shh-N), or anti-Flag or anti-Myc antibodies followed by protein A-Sepharose (for epitope-tagged rSmo or mPtc, respectively), and then separated on a denatured 8% SDS polyacrylamide gel. Proteins were detected either by exposure of the dried gel to film (for ¹²⁵I-Shh-N) or by blotting to nitrocellulose and probing with antibodies to Flag or Myc epitopes, using the ECL detection system (Amersham). To examine the interaction between rSmo and mPtc, cells were either lysed directly or after exposure to ¹²⁵I-Shh-N with or without an excess of unlabelled Shh-N (30 min at 37 °C, followed by 3 washes in phosphate buffered saline). Lysates were immunoprecipitated with antibodies against one of the epitope tags, subject to denaturing SDS-PAGE, and then resulting gels were either exposed to film (when ¹²⁵I-Shh-N was present) or transferred to nitrocellulose and analysed with antibody against the second epitope tag. For cross-linking, cells were resuspended in buffer containing 50 pM ¹²⁵I-Shh-N with or without a 1,000-fold excess of unlabelled Shh-N, and incubated for 2 h at 4 °C. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (10 mM) and *N*-hydroxysulphosuccinimide (5 mM) (Pierce) were added (30 min at room temperature), and the cells were washed 3 times with PBS. Cells were then lysed and protein complexes were immunoprecipitated with antibodies to the epitope tags as indicated, and run on a 4% SDS polyacrylamide gel. For equilibrium binding analysis, 293 cells were incubated with 50 pM ¹²⁵I-Shh-N and various concentrations of unlabelled Shh-N (Cold Ligand). The IGOR program was used to determine *K_d*.

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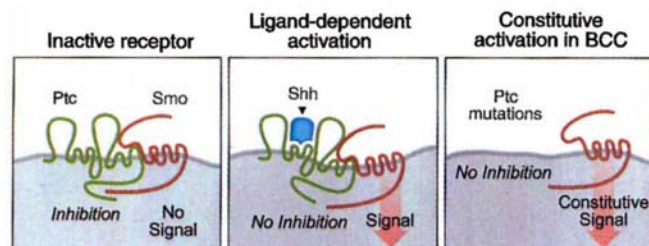


FIG. 6 Model describing the putative Shh receptor and its activation by Shh-N or following inactivation of Ptc. (Note that the actual binding site of Shh-N to Ptc and the interaction sites between Ptc and Smo have not been determined at the molecular level. In addition, the illustrated secondary structures of Smo and Ptc are based only on the primary structure of these proteins and are therefore hypothetical.)

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Structure of the complex between human T-cell receptor, viral peptide and HLA-A2

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Recognition by a T-cell antigen receptor (TCR) of peptide complexed with a major histocompatibility complex (MHC) molecule occurs through variable loops in the TCR structure which bury almost all the available peptide and a much larger area of the MHC molecule. The TCR fits diagonally across the MHC peptide-binding site in a surface feature common to all class I and class II MHC molecules, providing evidence that the nature of binding is general. A broadly applicable binding mode has implications for the mechanism of repertoire selection and the magnitude of alloreactions.

THE specificity of the T-cell response is determined by recognition of an antigenic peptide bound to a class I or class II MHC glycoprotein by the antigen-specific T-cell receptor (TCR). The TCR interaction initiates signals in T cells that, together with non-antigen-specific signals, trigger development of the T-cell repertoire, regulation of the immune response and activation of cytolytic T cells (CTL) (reviewed in ref. 1). X-ray crystal structures of several complexes of peptides with both class I and class II MHC molecules revealed how peptides are bound in a groove between two α -helices on the membrane-distal surface of MHC molecules (reviewed in refs 2, 3). MHC/peptide complexes present a compound surface composed of atoms of the peptide and the MHC molecule, providing a molecular mechanism for MHC-restricted antigen recognition by TCR^{4–8}.

Like antibody molecules, $\alpha\beta$ TCRs are encoded by a family of gene segments that rearrange to generate the diverse TCR repertoire, estimated to contain 10¹⁴ different sequences⁹. The first two variable loops (CDR1 and CDR2) of the human α - and β -chain are encoded by the 42 V α ¹⁰ and 46 V β genes¹¹; the third variable loops (CDR3) are encoded following the joining of V genes to one of a number of J-region and, in the case of V β , D and J-region gene segments. The joining reactions themselves generate further diversity both by removing nucleotides and by introducing non-germline bases ('N') at each junction.

Although the diversity-generating mechanism is very similar for antibodies and TCR, there are many more TCR J segments and N additions⁹, and apparently less variation at CDR1 and CDR2¹². These genetic differences suggest that TCRs differ structurally

from antibody fragments (Fabs) by having less difference in overall shape as a result of reduced diversity at variable loops 1 and 2, but more variation, due to increased loop-3 diversity, in the shape of the centre of the combining site, where in Fabs the third loops converge to form a pocket. Models of the interaction of TCR with MHC molecules have been proposed that orient the diverse CDR3 loops over the (diverse) peptides and the less diverse CDR1 and CDR2 loops over the correspondingly less diverse MHC molecules^{9,13–16}.

We have crystallized a ternary complex containing a soluble human $\alpha\beta$ TCR (A6)¹⁷ specific for the Tax peptide of the human T-cell lymphotropic virus HTLV-1, with that peptide bound to the human MHC molecule HLA-A2 (ref. 18). The TCR and HLA-A2 were both expressed as individual polypeptide chains in bacteria, refolded, and shown to retain the binding specificity of the molecules *in vivo*^{18,19}. The X-ray structure of the HLA-A2/Tax peptide has been determined previously and predicts peptide positions that may be recognized directly by TCR²⁰. HTLV-1 is associated with an adult T-cell leukaemia and a slowly progressive neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)²¹. A 40-fold higher frequency of HTLV-1-specific CTL, most of which are specific for the Tax peptide (LLFGYPVYV), is found in HLA-A2⁺ HAM/TSP patients relative to asymptomatic HTLV-1-infected individuals²².

The 2.6 Å structure of the TCR/Tax/HLA-A2 complex shows that the TCR is oriented diagonally over the MHC/peptide complex, with CDR1 and CDR3 from both V α and V β contacting the peptide. This orientation appears similar to that in the preliminary

TABLE 1 Structural analysis

Data sets	Resolution limit (Å)	Mosaicity	$R_{\text{merge}}^{\dagger}$	Comp $\ddagger$ (red)	I/σ_1	$R_{\text{deriv}}^{\ddagger}$ (sites)	$R_{\text{cullis}}^{\ddagger}$	$F_w/e^{\S}$	F.o.m. (res.)
C2 (Z = 1)*									0.41 (3.5 Å)
Native	2.90	2.5°	13.9	82 (3.7)	7.3				
L1C + EMP	3.10	1.0°	10.1	98 (2.5)	8.2	22.4 (1)	75	1.13	
K91C + EMP	3.20	2.4°	8.6	81 (2.7)	9.2	21.6 (1)	89	0.79	
Y67C/K91C + EMP	2.60	1.1°	10.9	95 (3.1)	7.0	27.2 (2)	85	0.94	
C2 (Z = 1)*									0.35 (3.5 Å)
Native	2.80	1.5°	11.8	79 (1.8)	6.4				
L1C + EMP	3.40	0.6°	10.7	75 (1.8)	6.3	23.5 (2)	76	0.94	
K ₉ PtCl ₄	3.60	1.4°	11.8	93 (2.5)	7.0	34.5 (5)	82	0.92	
P2 ₁ (Z = 2)*									0.30 (3.7 Å)
L1C/11C native	3.75	3.0°	14.3	86 (2.6)	6.6				
L1C/11C + EMP	3.40	2.5°	13.9	85 (2.6)	6.5	35.4 (2)	80	1.12	
Molecular replacements $\P$		HLA-A2/Tax9	TCR V α	TCR V β		TCR C β			
Rotation (10–3.8 Å)		7.3 σ	2.3 σ	4.3 σ		3.4 σ			
Translation (15–3.8 Å)		10.3 σ	8.5 σ	10.3 σ		5.7 σ			
Refinement resolution	Reflections (free)	Atoms	$R_{\text{free}}^{\ddagger}$	$R_{\text{cryst}}^{\ddagger}$	Bonds $\#$	Angles $\#$	B-factors**		
6.0–2.6 Å	26,273 (3,006)	5,716	32.2	24.0	0.013 Å	1.81°	4.8 Å ²		
Domains	$\alpha_1\alpha_2$	α_3	β_2m	Tax9	V α	V β	C β		
Mean B-factors	27 ± 15 Å ²	81 ± 32 Å ²	29 ± 16 Å ²	19 ± 7 Å ²	36 ± 15 Å ²	36 ± 15 Å ²	65 ± 18 Å ²		
Real-space fit $\ddagger\ddagger$	0.87 ± 0.03	0.74 ± 0.15	0.86 ± 0.09	0.89 ± 0.02	0.87 ± 0.05	0.88 ± 0.03	0.80 ± 0.12		

* Space groups of crystal forms and derivatives are described in Methods. Z, number of MHC/Tax/TCR complexes per asymmetric unit.

$\dagger R_{\text{merge}} = 100 \times \frac{\sum_h \sum_j |I_{h,j} - \bar{I}_h|}{\sum_h \sum_j I_{h,j}}$, where I_h is the mean intensity of symmetry-related reflections, $I_{h,j}$.

$R_{\text{der}} = 100 \times \frac{\sum_{hkl} |F_p - F_{ph}|}{\sum_{hkl} F_p}$, where F_p is the native amplitude and F_{ph} is the derivative amplitude. Sites, number of heavy atom sites per asymmetric unit.

$R_{\text{cullis}} = 100 \times \frac{\sum_{hkl} |F_p \pm F_{ph} - F_{\text{calc}}|}{\sum_{hkl} |F_p \pm F_{ph}|}$, where F_p is the native amplitude, F_{ph} is the derivative amplitude for centric reflections, and F_{calc} is the the calculated heavy-atom structure factor.

$R_{\text{free}} = 100 \times \frac{\sum_{hkl} |F_{\text{obs}} - F_{\text{calc}}|}{\sum_{hkl} F_{\text{obs}}}$, where R_{free} is calculated for a randomly chosen 10% of reflections ($F > 0$) omitted from refinement, and R_{cryst} is calculated for the remaining 90% of reflections ($F > 0$) included in refinement.

$\ddagger$ Comp = $100 \times (\text{number of observed unique reflections})/(\text{number of possible unique reflections})$; red = $(\text{number of observed reflections})/(\text{number of observed unique reflections})$.

$\S F_w/e$ is the phasing power, with F_h being the mean amplitude of the heavy-atom structure factor and e the r.m.s. lack-of-closure error.

|| F.o.m., figure of merit; res., resolution to which figure of merit is determined.

$\P$ Rotation and translation solution peak heights above the mean in rotation and translation function maps.

$\#$ r.m.s. deviations from ideal values.

** r.m.s. deviation between B-factors of bonded atoms.

$\ddagger\ddagger$ Real-space fit correlation coefficient⁴⁶ calculated from SIGMAA-weighted⁴⁴ $2F_{\text{obs}} - F_{\text{calc}}$ electron density and the refined model of MHC/Tax/TCR.

description of a mouse TCR (2C)/peptide/H-2K^b complex³¹. All three variable loops of V α and CDR3 of V β contact conserved and polymorphic positions on the α -helices of the MHC molecule. Evidence is presented that the observed binding mode is general for human and mouse class I and class II molecules. The implications of the size and shape of the TCR/peptide/MHC interaction for the specificity of TCRs and mechanisms of immunological tolerance are discussed.

Overview of the intercellular complex

The crystal structure was determined using isomorphous replacement, molecular replacement, iterative real-space averaging between crystal forms and around a non-crystallographic symmetry axis, and density modification (see Methods). All of the crystals exhibited large mosaic spreads, affecting the quality of data that could be collected (Table 1). The interface between TCR and MHC/peptide is in unambiguous electron density, as are the α_1 , α_2 , and β_2 -microglobulin (β_2m) domains of the MHC and the V α

and V β domains of the TCR. Regions distal to the interface between the MHC and TCR appear to be mobile and disordered. (Note the increase in the magnitude of temperature factors with increasing distance from the interface; Fig. 1 and Table 1.) Parts of the HLA-A2 α_3 domain and some loops of TCR C β are unclear (see Methods). Although several strands and side chains of TCR C α near C β are visible, most of C α is disordered. The C α domain is not included in the final model, with little effect on the crystallographic residual (Methods and Table 1).

Figure 1 shows the TCR/Tax/HLA-A2 complex and how the TCR (top) and MHC molecule (bottom) would span the space between a CTL and virally infected target cell.

Orientation of TCR variable loops

When the peptide-binding site of HLA-A2 is viewed from above, the TCR is oriented diagonally across the site (Fig. 2a, b). The TCR third variable loops of both V α and V β meet at the centre of the binding site over the centre peptide residue Y5 (Fig. 2a). The

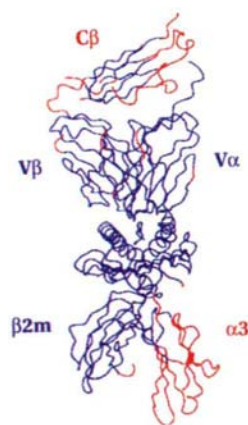


FIG. 1 Intercellular complex of A6 $\alpha\beta$ TCR (top) and the HTLV-1 Tax peptide bound to HLA-A2 (bottom) shown as an α -carbon trace, coloured by temperature factor⁴⁶: blue is low-temperature factor, and red is high-temperature factor ($>60 \text{ \AA}^2$) and shows less ordered regions. The TCR $C\alpha$ domain is not shown (see Methods).

CDR1 loops are also positioned over the peptide. CDR1 α extends from the peptide N terminus to Y5, and CDR1 β is over the C-terminal end of the peptide near Y8 (Fig. 2a). The second variable loops are over the MHC molecule, CDR2 α over the α 2 domain α -helix and CDR2 β over the α 1 domain α -helix.

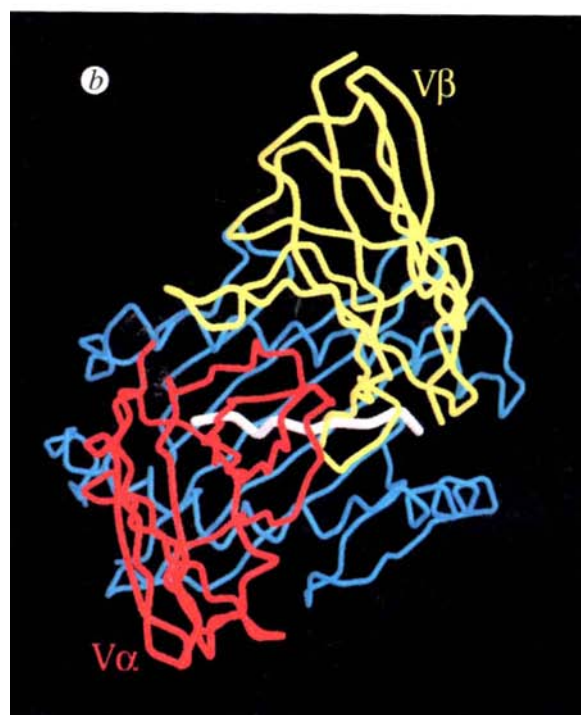
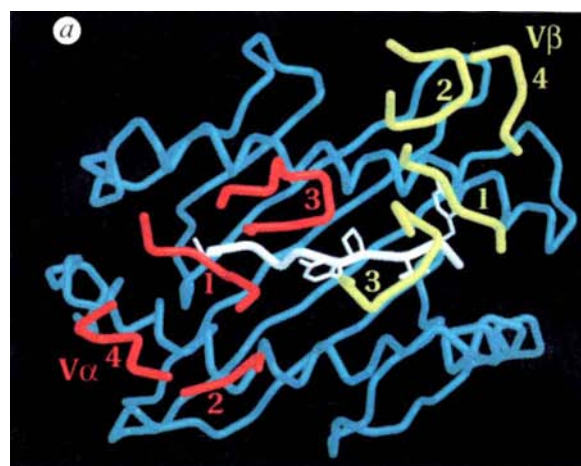
Although the location of the CDR3 loops over the peptide with CDR3 β toward the C terminus (Y8) and CDR3 α near the centre of the peptide (Y5) is consistent with mutation data^{15,16,23,25}, the diagonal orientation of the TCR over the binding site is not as originally predicted^{9,13,14,24}. Nathenson and colleagues²⁵, testing mutant mouse class I molecules against a panel of CTL, provided evidence for a diagonal orientation for TCR binding to H2-K^b like that observed here (although some of their mutations are too distant from the Tax TCR to be contacted and may have exerted their effects through distortions to the peptide). Subsequent mutation data on the class II molecule I-A^k (ref. 23), as well as the preliminary description of the mouse TCR (2C)/peptide/H-2K^b structure³¹, support that orientation.

Almost the entire Tax peptide is buried in the TCR/MHC interface (Fig. 2c). The TCR makes contacts with peptide residues L1, L2 and from G4 to Y8. Y5 is bound in a deep pocket at the centre of the TCR where the CDR3 loops converge (Fig. 2d, f), reminiscent of the structures of peptides complexed with anti-peptide Fabs (reviewed in ref. 26). The peptide is bound much more deeply in the MHC molecule (Fig. 2e) than it is in the TCR (Fig. 2d).

The diagonal orientation of the TCR allows the flat surface of the TCR (Fig. 2f) to interact with the peptide by fitting down between the two highest points on the MHC molecule, toward the N-terminal of the α 1 domain α -helix (top left, Fig. 2g) and towards the N terminal of the α 2 domain α -helix (bottom right, Fig. 2g), a feature of the diagonal orientation also recognized by Nathenson and colleagues²⁵. The high points of the α 2 domain α -helix (right, front, Fig. 2e) and the α 1 domain α -helix (left, back, Fig. 2e) are a topographical feature common to all class I and class II molecules.

Interaction with Tax and HLA-A2

Variable loops 1 and 3 of both V α and V β contact the Tax peptide, whereas loops 1, 2 and 3 of V α and loop 3 of V β contact the HLA-A2 α -helices (Fig. 3a, Table 2). The loop with the highest potential diversity, CDR3 β , extends across the site contacting the α 1 domain α -helix at residue 72, peptide residues Y5, P6, V7 and Y8, and ends by contacting the α 2 domain α -helix at residues 149, 150, 151 and 155 (Fig. 3a). Residues 95 and 101–103 of this loop form part of the central pocket in the TCR that surrounds Y5 and the central residues of the peptide (Fig. 3b). CDR3 α forms most of the other side of this central pocket, burying residues G4 and Y5 and the α 1 domain α -helix at residues 65, 66, 68 and 69 (Fig. 3a). Serine 31 from CDR1 α completes the central pocket, forming a hydrogen bond to the hydroxyl of peptide tyrosine, Y5. CDR1 α is positioned over the N terminus of the peptide and contacts the



α -helices of both the α 1 and α 2 domains (Fig. 3a), making a footprint comparable to that made by CDR3 α (Fig. 3c). One residue of CDR1 β , E30, contacts the peptide, forming a hydrogen bond to the Y8 hydroxyl group at the opposite end of the site. CDR2 β , although positioned over the α 1 domain, does not make any contacts; CDR2 α over the α 2 domain contacts helix residues 155, 158 and 166 (Fig. 3a).

A total of $998 \text{ \AA}^2$ of solvent-accessible surface area of HLA-A2/Tax is buried in the interface with TCR, including parts of five polymorphic positions on HLA-A2 (open circles in Fig. 3a) that could provide allelic specificity, and eleven positions conserved in human class I molecules (solid circles in Fig. 3a) that could provide a common binding site for TCR. Of 20 hydrogen bonds between TCR and MHC/peptide, eight are to conserved and three to polymorphic class I MHC residues, and nine are to Tax peptide. About $326 \text{ \AA}^2$ of peptide surface is buried by the TCR, two-thirds of the area buried in the average anti-peptide antibody interface ($420\text{--}620 \text{ \AA}^2$)²⁶. The surface of HLA-A2 buried by the TCR is $671 \text{ \AA}^2$, at the low end of the range for protein antigen/antibody interfaces ($620\text{--}899 \text{ \AA}^2$)²⁷. Overall, the buried surface of the peptide/MHC ($998 \text{ \AA}^2$) and TCR ($1,011 \text{ \AA}^2$) is only slightly larger than typical antigen/antibody interfaces²⁷. Over half of the

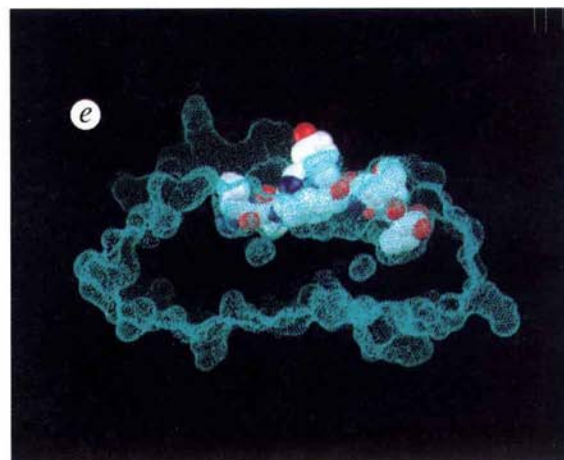
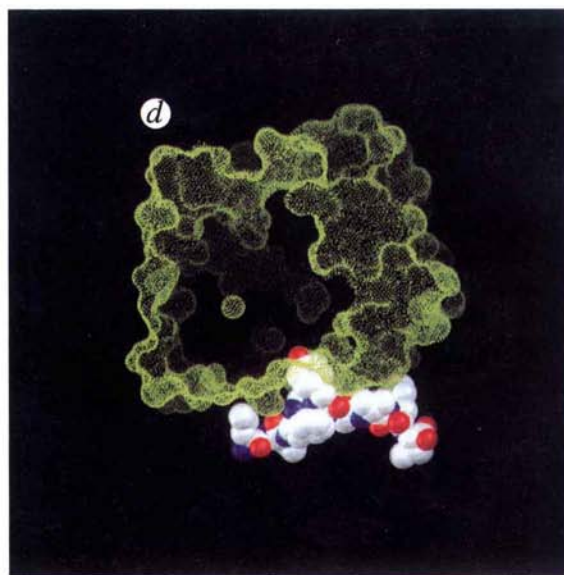
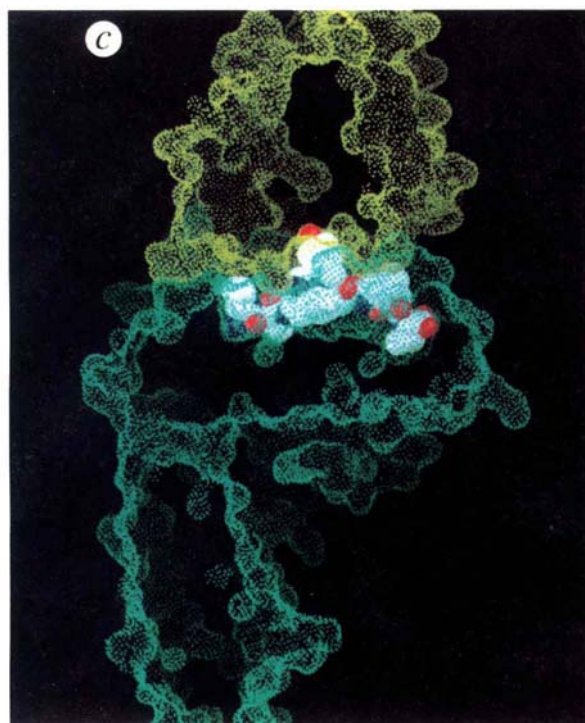
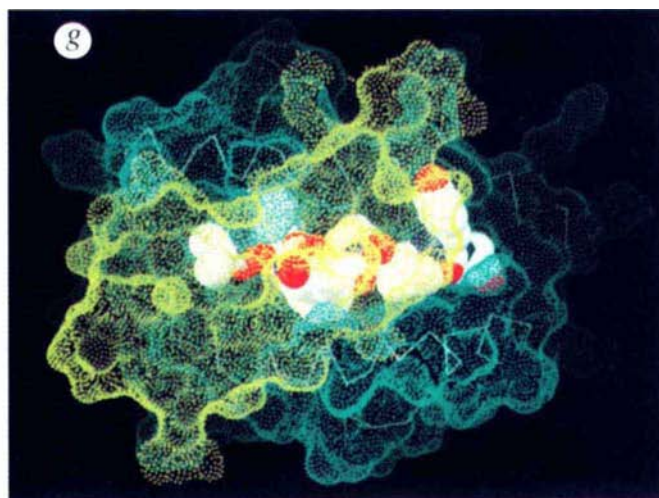
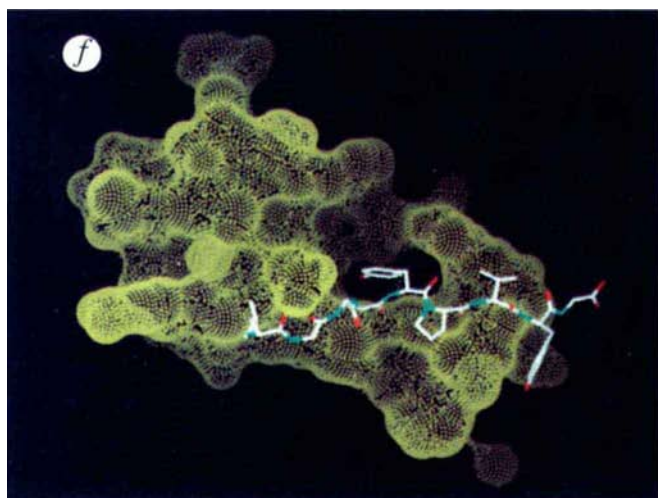


FIG. 2 Orientation of TCR on HLA-A2/Tax. *a*, Variable loops of TCR V α (red) and V β (yellow), Tax peptide (white), and HLA-A2 (blue) shown as an α -carbon trace. Also shown are side chains of the peptide contacted by TCR. *b*, TCR V α (red) and V β (yellow) domains bound to Tax/HLA-A2. *c*, Tax peptide is buried between the TCR and HLA-A2. The solvent-accessible surface (dot) of TCR V α and V β (yellow), HLA-A2 (blue) and Tax peptide (CPK model) is shown. Surfaces were calculated with a 1.4 Å probe radius⁵⁰. *d*, Interactions of bound Tax peptide with TCR V α and V β . *e*, Interactions of bound Tax peptide with HLA-A2. *f*, Flat TCR surface with central pocket. Bound Tax peptide is shown with Y5 in the pocket and Y8 at the edge of the flat surface. *g*, TCR surface (yellow) in contact, diagonally, with HLA-A2 (blue) and Tax peptide. Figures were made using the program MIDAS⁵⁰.



peptide surface buried by the TCR in the interface is covered by the most diverse loop, CDR3 β . CDR1 α and CDR3 α each contribute about 25%. Over half of the MHC surface buried by TCR is also covered by CDR3 loops and the remainder is covered about equally by CDR1 α and CDR2 α (Fig. 3c).

Only one residue of loop 4, (HV4) of the TCR, α 68K, contacts the MHC molecule, forming hydrogen bonds to T163 and E166, an acidic side chain conserved in mouse and human class I (corresponding to a conserved histidine in class II) (Fig. 3a).

Changes in interface conformation

A number of small adjustments of MHC and peptide main-chain and side-chain positions are found by comparing the Tax/HLA-A2

structure²⁰ to the TCR/Tax/HLA-A2 structure. The peptide appears to be pushed down further into the MHC binding site by the TCR; proline at position 6 (P6) is pressed down, and the side chain of valine 7 has moved up (Fig. 3d). If P6 had not moved it would have overlapped the positions of TCR residue 100 of CDR3 α and residue 98 of CDR3 β . MHC side chains appear to have moved to achieve close fits in the interface, and a small but significant shift in the MHC main chain is evident (Fig. 3d).

The structure of the uncomplexed A6 TCR is not known, but comparison of the TCR in this complex with the coordinates of the free TCR β chain²⁸ and the TCRV α dimer²⁹ indicates that the overall conformations of the CDR1 and CDR2 loops are essentially the same in these bound and unbound TCRs. The more

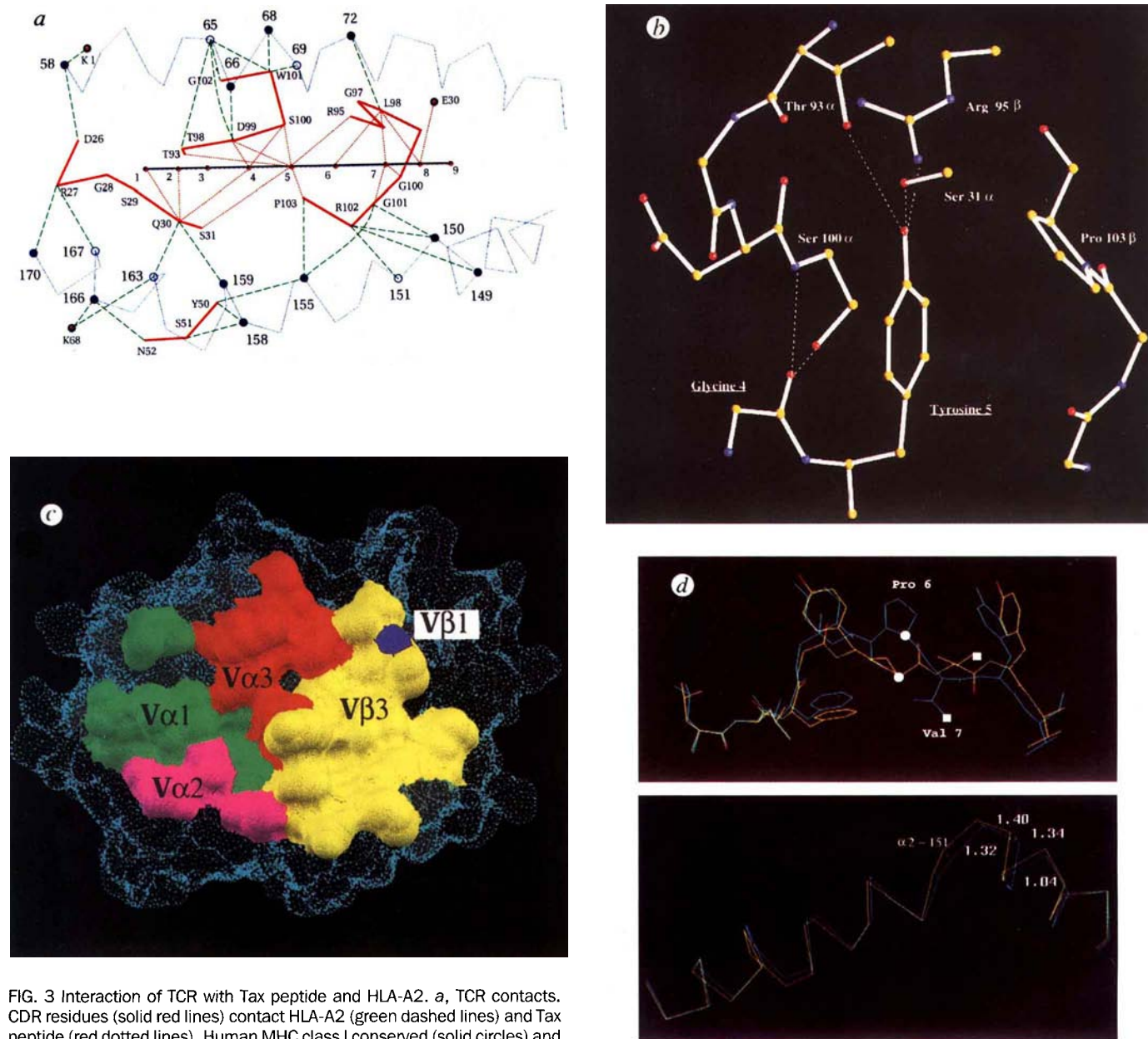


FIG. 3 Interaction of TCR with Tax peptide and HLA-A2. *a*, TCR contacts. CDR residues (solid red lines) contact HLA-A2 (green dashed lines) and Tax peptide (red dotted lines). Human MHC class I conserved (solid circles) and polymorphic (open circles) residues are shown. D26–S31, CDR1 α ; Y50–N52, CDR2 α ; T93–G102, CDR3 α ; E30, CDR1 β ; R95–P103, CDR3 β . TCR (V α 2.3[AV2S1A2]–J α 24–C α , V β 12.3[BV13S1]–D β 2.1–J β 2.7–C β 2)¹⁸ (numbered according to ref. 51). CDR3 residues contacting MHC and peptide are encoded by N addition (α 93) and J segment for V α and N addition (β 95, β 102, β 103) and D segment for V β . For clarity, the peptide is depicted as a straight line and contact between V α 30Q and HLA-A2 66 is not shown. *b*, TCR pocket around Gly 4 and Tyr 5. Figure was made using RIBBONS software⁵². *c*, MHC/peptide solvent-accessible surface buried by

the CDRs of the TCR, coloured according to contact region. *d*, Conformational differences of peptide (top) and MHC α 2 domain helix (bottom) in the TCR/Tax/MHC structure (yellow) and the Tax/MHC structure (blue). The Pro 6 C α (dot) has moved 2.7 Å and the Val 7 C γ (square) 4.6 Å. Helix displacements as measured between α -carbons (α 2 148–151) are shown in Ångströms (a and c were produced with MIDAS⁵⁰, d with O⁴⁶).

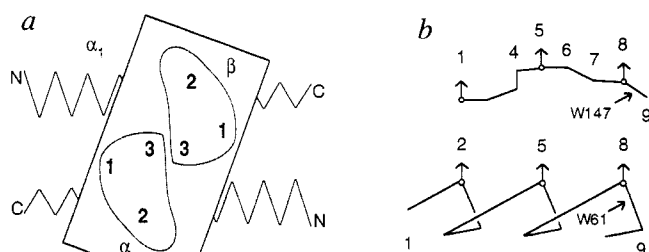


FIG. 4 Diagonal TCR binding would cover peptides bound to class I and class II MHC molecules. *a*, TCR $\alpha\beta$ and CDR loops (numbered) shown diagonally between the N-terminal peaks of the helical regions of MHC molecules. *b*, Side-chain locations of bound peptides (side view). Peptides bind to class I molecules as extended chains with a kink (top) and to class II MHC molecules as an extended polypyrrolone type-II conformation, with peptide side chains projecting every 120° along the chain (triangles, bottom). In both classes of MHC molecule, the carbonyl oxygen of the peptide residue 8 is hydrogen-bonded to a tryptophan (W) residue conserved in all class I (top) and class II (bottom) molecules at a homologous location of the lower helix in *a*^{40,53}.

variable CDR3 loops have different conformations, as predicted from Fab structures.

TCR structure compared with Fab

The overall structure of the TCR in the TCR/Tax/HLA-A2 complex is similar to the structure of a Fab. The $V\alpha$ - $V\beta$ interface buries about $1,575 \text{ \AA}^2$ ($\sim 780 \text{ \AA}^2$ from each domain), forming a contact surface that is among the largest observed in Fabs³⁰. This suggests that the particular $V\alpha V\beta$ combination in the A6 TCR makes a strong V-V contact that is unlikely to have rearranged upon binding antigen, like the induced-fit seen in a Fab with a small V-V interface ($1,063 \text{ \AA}^2$)³⁰, and discussed with respect to the small V-V interface in 2C TCR³¹. The disorder of the $C\alpha$ domain in crystals of the complex precludes detailed assessment of the $C\alpha$ - $C\beta$ interface, although it is clear from the electron density that it comprises a large area of residues (see Methods). The $C\alpha$ domain determined in a mouse TCR (2C) has an unconventional fold³¹. The absence of the TCR carbohydrate that is found at the $C\alpha$ - $C\beta$ interface and of the interchain disulphide bond³¹ may contribute to the disorder observed.

The 'elbow angle' between $V\beta$ and $C\beta$ is approximately the same in the TCR/Tax/HLA-A2 complex as in the free β -chain structure²⁸, the 2C TCR³¹, and within the range found in Fabs³². The $V\beta$ - $C\beta$ interface in the complex buries about $800 \text{ \AA}^2$, similar to the free β -chain²⁸ and 2C TCR³¹ structures, and about twice the area of this interface in Fab structures³².

TCR signalling

No gross conformational change of the TCR was observed in the complex relative to the structures of isolated TCR domains or Fabs which might indicate that it could send information to the cytoplasm that it had bound antigen. Indeed, no overall conformational change has ever been observed in Fabs that correlates with antigen binding, even when the V domains rotate up to 16° as a result of induced fit in the combining site³⁰. The high-temperature factors in $C\alpha$ of an uncomplexed TCR³¹ suggest that flexibility is a feature of this area of isolated, soluble $\alpha\beta$ TCR, consistent with the TCR/Tax/HLA-A2 structure. It may be necessary to include CD3 and ζ -chains in crystalline complexes to be able to observe relevant conformational changes, if they exist.

The current (and preliminary) absence of structural evidence for a TCR conformational change in TCR/peptide/MHC that might initiate signalling, favours signalling mechanisms based on the assembly of complexes following TCR engagement³³, or on segregation of engaged TCRs in cell-cell attachment patches^{34,35}.

Specificity and tolerance

The small size of the contact between the TCR and the Tax peptide, $326 \text{ \AA}^2$, and the observation that although substantial contacts are made to peptide residues Y5 and Y8, only a few atoms of peptide residues 1, 2, 4, 6 and 7 are in contact, places physical limits on TCR specificity for peptide. For example, when Y5, which is bound in the central pocket of the TCR, is substituted

to alanine, the peptide still forms A6 TCR/peptide/HLA-A2 complexes that crystallize and is a partial agonist¹⁷. On the basis of the TCR/Tax/HLA-A2 structure, it seems likely that other peptide sequences will be able to satisfy the twin requirements of binding to HLA-A2 and to the A6 TCR. How many of those sequences might be expressed and presented in humans? (see ref. 36 for example). Limited TCR specificity would favour tolerance mechanisms that depend on the context of antigen presentation, including other receptors and adhesion molecules, to restrict the potential for cross-reaction^{37,38}.

Generality of the binding mode?

The diagonal binding observed in the TCR/Tax/HLA-A2 complex appears to result from properties shared by all classical class I and class II MHC molecules and probably all $\alpha\beta$ TCRs. The A6 and the 2C³¹ $\alpha\beta$ TCRs, like Fabs that bind protein antigens³⁹, have a relatively flat contact surface formed by the CDR loops with a central pocket where the CDR3 loops converge (Fig. 2f). The top surface of class I and class II MHC molecules is not flat, but has two 'peaks' near the N termini of the α -helical regions (top left and bottom right in Fig. 4a); peptides bind to MHC molecules low in the groove between these α -helices (Fig. 2e). For the fairly flat surface of the TCR to fit at a low enough point on the MHC surface to contact the length of exposed peptide, a diagonal orientation is required. Either significant translations along the peptide binding site or rotations from the observed orientation would break this overall shape complementarity. Contacts between TCR and the α -helical 'peaks' are observed (Fig. 3a), but result from residues at the edges and sides of the flat TCR surface.

The peptide residues most exposed on class II MHC complexes, positions 2, 5 and 8, align closely with positions 1, 5 and 8 which the TCR covers in the Tax peptide bound to HLA-A2, despite the differences in the conformations of peptides bound to the two types of MHC molecules (see Fig. 4 of ref. 3) (Fig. 4b). The TCR binding mode observed here and with mouse TCR 2C³¹ covers the length of the peptide with the central TCR pocket positioned over the middle of the peptide. It would also cover a peptide on a class II molecule from near pocket 1 to 9⁴⁰.

The suggestion that a diagonal orientation allows a general 'interlocking' binding mode between TCR and MHC was first proposed on the basis of mutations on mouse class I molecules²⁵. Mutation data from class II mouse molecules support the same mode of binding²³, as does the preliminary description of a mouse TCR/peptide/H-2K^b complex³¹. In addition to the structural arguments presented here, functional arguments have been made in favour of a general binding mode (refs 23, 41 for example). For example, it would provide a molecular mechanism for an inherent bias of TCR for self-MHC⁴² and, coupled with the physical limit seen on TCR specificity for peptide, helps to explain the ability of one peptide positively to select a nearly normal TCR repertoire^{41,43}. A general TCR binding mode would simplify the processes of selection during repertoire development and provide

TABLE 2 Contacts between TCR and MHC peptide

CDR1 α	α 1 K	N	*	HC58 E	OE2
	α 26D	OD2	*	HC58 E	OE1
	α 27 R	CG		<u>HC167 W</u>	CZ2
		NE		HC170 R	NH1
	α 30 Q	NE2		HC66 K	NZ
		NE2		Tax1 L	CD2
		NE2	*	Tax2 L	O
		OE1	*	Tax4 G	N
		OE1		HC159 Y	CD1
		CG		<u>HC163 T</u>	OG1
CDR2 α	α 31 S	OG	*	Tax5 Y	OH
	α 50 Y	CG		HC155 Q	OE1
		O		HC158 A	CB
	α 51 S	OG		HC158 A	CB
HV4 α	α 52 N	ND2	*	HC166 E	OE2
	α 68 K	NZ	*	HC166 E	OE1
		NZ	*	<u>HC163 T</u>	OG1
CDR3 α	α 93 T	OG1	*	Tax5 Y	OH
	α 98 T	OG1	*	<u>HC65 R</u>	NH2
	α 99 D	OD2	*	<u>HC65 R</u>	NE
		CB		HC66 K	CG
		CA		Tax4 G	O
		O		Tax5 Y	OH
	α 100 S	N	*	Tax4 G	O
		OG	*	Tax4 G	O
		CA		Tax5 Y	CE1
	α 101 W	CZ3		<u>HC69 A</u>	CA
		CZ3		HC68 K	C
		O		<u>HC65 R</u>	CD
	α 102 G	CA		<u>HC65 R</u>	NE
	β 30 E	OE2	*	Tax8 Y	OH
CDR1 β	β 95 R	NH2	*	Tax5 Y	OH
	β 98 L	CD1		Tax6 P	O
CDR3 β		CB		Tax8 Y	CZ
		O	*	Tax8 Y	N
		O		Tax7 V	CA
		CD2		HC72 Q	CG
	β 100 G	C		Tax7 V	CG2
	β 101 G	N	*	HC150 A	O
		O	*	HC155 Q	NE2
		N		Tax7 V	CG2
	β 102 R	NH1	*	HC149 A	O
		NE	*	HC150 A	O
		CD		<u>HC151 H</u>	CD2
	β 103 P	CB		Tax5 Y	OH
		CD		HC155 Q	NE2

Asterisks denote hydrogen bonds (2.5–3.4 Å acceptor–donor distance). Unstarred residues, van der Waals contacts (<4.0 Å); only closest two atoms tabulated. α and β , TCR; HC, conserved class I MHC heavy-chain residues; underlined HC, polymorphic heavy-chain residues.

an explanation for the magnitude of the allereactive response. One way that the danger inherent in hypervariable molecules may be controlled is by restricting their opportunity for cross-reactivity to a single binding mode on MHC molecules, which could be part of the basis of mechanisms that maintain immunological tolerance.

Note added in proof: K. C. Garcia and I. A. Wilson kindly provided the coordinates of the free, uncomplexed 2C TCR after this manuscript was submitted, permitting the disorder of the A6 C α domain to be confirmed. Including in refinement the 2C C α domain fitted to the A6 TCR/Tax/HLA-A2 electron density gave no improvement in the R_{free} , even at low resolution. $\square$

Methods

Data collection and processing. Crystals with space groups C2 ($a = 229.3$ Å, $b = 49.5$ Å, $c = 96.0$ Å, $\beta = 89.6^\circ$)¹⁸ containing one complex per asymmetric unit and $P2_1$ ($a = 229.9$ Å, $b = 49.5$ Å, $c = 96.0$ Å, $\beta = 88.9^\circ$) with two complexes per asymmetric unit, were soaked in 14% PEG 8000, 100 mM

Mg(CH₃COO)₂, 50 mM Tris, pH 8.5, containing 20% glycerol for 2 min, mounted in a fibre loop, and flash-cooled in liquid N₂. X-ray data were collected on an ADSC 1K CCD detector (1° – 2° oscillations, CHESS A1 beamline, $\lambda = 0.9$ Å, 100 K). All crystals exhibited large mosaic spread ($\sim 1.5^\circ$) and anisotropic diffraction (Table 1). Data were processed with DENZO, SCALEPACK (Z. Otwinowski, personal communication), and TRUNCATE⁴⁴. Two forms of the C2 crystal (A and B) exist with identical cell constants but slightly different molecular orientations ($R_{\text{deriv}} = 40\%$; average 2° and 3° difference in some domains).

Molecular replacement. The position of HLA-A2/Tax-peptide²⁰ was located with AMORE⁴⁴ in crystal A. TCR 1934.4 V α ²⁹ and TCR 14.3.d β -chains²⁸, with side chains differing from A6 TCR made alanine and V α CDRs and the C β 131–142 loop omitted, were also located ($R_{\text{cryst}} = 43\%$, 6–3.5 Å). With HLA-A2/Tax peptide fixed, the V β , C β and V α positions were determined successively, each using the preceding domains. After density modification by solvent flattening or flipping and histogram matching⁴⁴ using envelopes calculated with ENVAT (D. Madden, personal communication), side chains unique to A6 TCR were identifiable but as the interface between HLA-A2/Tax and TCR was ambiguous, heavy-atom derivatives were used.

Multiple isomorphous replacement. Crystals with cysteine substitutions in the Tax peptide, L1C and L1C/L11C (containing L1C and the C-terminal additions G10 and C11), and in β_2 m (K91C and double mutant Y67C/K91C) were soaked in ethyl mercury phosphate (EMP) and yielded derivatives. L1C/L11C crystallized in space group $P2_1$, breaking the centring of the C2 group, resulting in two complexes per asymmetric unit. K₂PtCl₆ was used previously for solving HLA-A2 (ref. 4). Crystals were soaked for 4–24 h in 1 mM compound. For mercurials, crystals were presoaked in buffer containing 1–2 mM dithiothreitol for 4 h. The mercury positions in L1C in form A and form B crystals were found by difference-Patterson syntheses. Other heavy atoms were located by difference Fourier syntheses using molecular replacement (MR) phases (Table 1). Heavy-atom parameters were refined with VECREF and MLPHARE⁴⁴ and re-refined with protein phases after density modification.

Iterative real-space averaging. Averaging between form A and form B crystals and fourfold averaging among form A, form B, and the two complexes in the $P2_1$ crystal were carried out to 3.5 Å with MAVER⁴⁵ or DMMULT⁴⁴. Seven-domain envelopes (for α 1 α 2/Tax peptide, α 3, β_2 m, V α , C α , V β and C β) were constructed with ENVAT and transformations for each were derived from MR or from electron density correlations. Electron density from MIR phased maps, MIR/MR phase-combined maps, MR phased or MIR/model phase-combined maps were iteratively averaged. The electron density after averaging allowed an unambiguous definition of the CDR loops and TCR to MHC/peptide contacts.

Model building and refinement. The mercury derivative of Y67C/K91C, which diffracted best, was used for building⁴⁶ and refinement (Table 1) essentially as described⁴⁷ with XPLOR⁴⁸ and REFMAC⁴⁴, using a maximum-likelihood target in the latter case. An anisotropic B -factor tensor ($B_{11} = -6$ Å², and $B_{22} = B_{33} = 6$ Å²) determined from Wilson plots and a bulk solvent correction were applied^{44,48}; data from 12 to 2.6 Å were included. Simulated annealing omit maps⁴⁸ were used extensively. The α 3 domain of HLA-A2, which forms no crystal contacts, is disordered. Refinement at low resolution (12–6 Å) with and without α 3, indicated that α 3 makes little coherent contribution to the diffraction. As the α 3 domain has previously been refined to high resolution⁴⁹, the model was maintained in refinement, although some regions (190–202, 217–227, 246–259, 267–268) have discontinuous density and high temperature factors. The C α domain of TCR also appears to contribute little to coherent scattering even at low resolution. It has not been modelled, although several strands and side chains proximal to C β are visible. The model contains: residues 1–274 of heavy chain and 0–99 of β_2 m of HLA-A2; 1–9 of Tax peptide; 1–122 of TCR α ; 3–246 of TCR β , except for three loops (131–144, 180–189, 220–229); two ethyl mercury groups and 37 waters. One water coordinates TCR residues at the interface (V α 101, V β 30, 50, 98), and three waters contact Tax peptide. Breaks in the main chain are in the V β –C β connection (117–118) and in C β (199–201, 207–208). Side chains of 47 residues have been omitted from later stages of refinement (mainly in α 3) because they appear to be disordered. Only one, TCR α 1 (lysine) is near the interface (Fig. 3a). No residues have disallowed ϕ , ψ angles; 86% of residues have favoured angles.

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CORRESPONDENCE and requests for materials should be addressed to D.C.W. Coordinates will be deposited in the Protein Data Bank (Brookhaven National Laboratory, Upton, New York) and are available before their release by e-mail (ghosh@crystal.harvard.edu).

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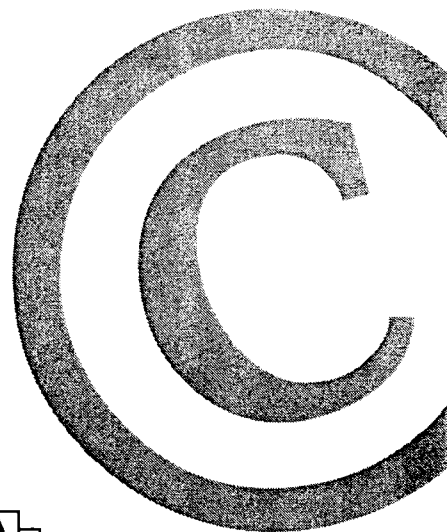
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Geometry and physics of knots

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KNOTS are usually categorized in terms of topological properties that are invariant under changes in a knot's spatial configuration^{1–4}. Here we approach knot identification from a different angle, by considering the properties of particular geometrical forms which we define as 'ideal'. For a knot with a given topology and assembled from a tube of uniform diameter, the ideal form is the geometrical configuration having the highest ratio of volume to surface area. Practically, this is equivalent to determining the shortest piece of tube that can be closed to form the knot. Because the notion of an ideal form is independent of absolute spatial scale, the length-to-diameter ratio of a tube providing an ideal representation is constant, irrespective of the tube's actual dimensions. We report the results of computer simulations which show that these ideal representations of knots have surprisingly simple geometrical properties. In particular, there is a simple linear relationship between the length-to-diameter ratio and the crossing number—the number of intersections in a two-dimensional projection of the knot averaged over all directions. We have also found that the average shape of knotted polymeric chains in thermal equilibrium is closely related to the ideal representation of the corresponding knot type. Our observations provide a link between ideal geometrical objects and the behaviour of seemingly disordered systems, and allow the prediction of properties of knotted polymers such as their electrophoretic mobility⁵.

Although unique geometrical forms of knots have been discussed previously^{6–9}, an analytical solution for the minimal length-to-diameter ratio for a given knot is not yet known. To model ideal representations, we modified the Metropolis Monte Carlo procedure used previously for the simulation of DNA chains^{10–12}. A chain of 160 segments of equal length was closed into a given type of knot and a virtual, impenetrable tube with uniform diameter was centred around the axial path of the knot. The diameter of the tube was then expanded uniformly until first contact occurred between portions of the tube surrounding non-neighbouring segments of the knot. New configurations of the knot were then generated by random crankshaft rotations of chain segments to find configurations which allow maximization of the diameter of the tube. To avoid change of knot type during simulations we verified that their Alexander polynomial^{1,13} remained the same. This polynomial is calculated by a specific scoring of crossings in a given planar projection of the axial trajectory of the knot; it is independent of the knot's actual shape. However, different knots can (very rarely) have the same Alexander polynomial. To further reduce the possibility that crankshaft rotations of sections of phantom chains could lead to a change between two different knot types with the same Alexander polynomial, we visually inspected the final configurations to ensure that the minimal number of crossings did not change.

The modelled knots usually 'flowed' into their ideal geometrical representations, repeatedly adopting almost identical configurations, where such characteristic properties as length/diameter ratio and average crossing number of the axial trajectory did not

vary by more than 1.0%. We have computed the ideal configurations of 21 different knot types (Table 1).

Some of the ideal representations of the 21 knot types are shown in Fig. 1. The designation of the knots in Table 1 and Fig. 1 corresponds to the designation in standard tables of knots¹⁴. The first number indicates a minimal crossing number in a planar projection for a given type of knot, and the subscript index number indicates the tabular position of this knot type among the knot types with the same minimal number of crossings. The knots denoted as 'square' and 'granny' represent composite knots, which are not listed in standard tables, as they may be formed by joining two simpler knots. To check our algorithm's ability to handle more complicated knots we turned to famous Perko pair. These two representations of 10 crossing non-alternating knots

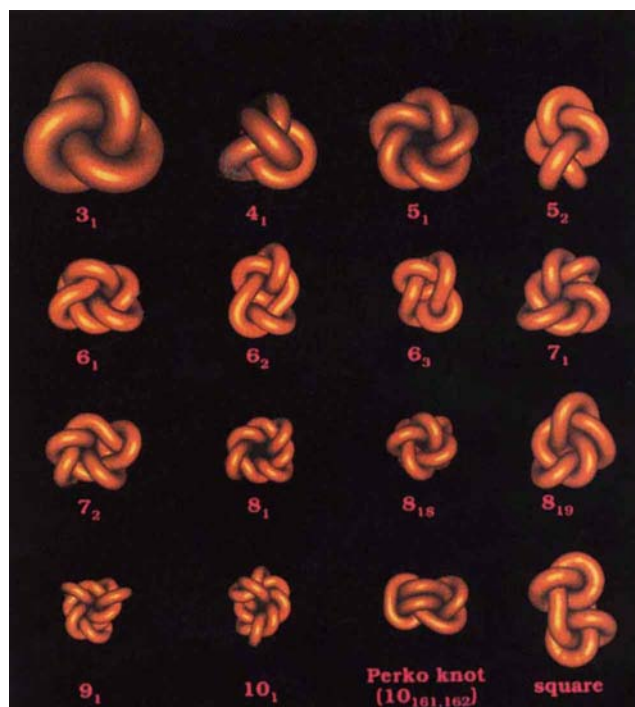


FIG. 1 Ideal geometrical representations of various types of knots. The designations of prime knots follow the standard nomenclature for knots. The square knot, an example of a composite knot, can be obtained by tying two trefoils of different handedness on the same piece of string (the 'granny' listed in Table 1 is composed of two trefoils of the same handedness). Notice that the overall dimensions of knots with the same axial length decrease with increasing complexity of knots. Interestingly, the non-alternating knot 8_{19} has dimensions typical for knots with seven crossings as if non-alternation allows for less constraint on the axial path than alternation. Also, the Perko knot (a non-alternating knot with ten crossings in its tabular representation) has dimensions typical for knots with nine crossings in their standard tabular representation. The square knot belongs to knots with six crossings and its dimensions correspond to knots with this crossing number. The ideal configurations shown were obtained from computer simulations where configurations were randomly distorted and the rules of Metropolis *et al.*¹⁹ were applied to search for the configuration which allows maximalization of diameter of a virtual cylinder centred at the axis of the knot. A new trial configuration was always accepted if it allowed the diameter ϕ of the virtual tube to increase, but when it led to a decrease of the diameter the new trial configuration was accepted with a probability equal to $\exp((\phi_{\text{new}} - \phi_{\text{old}})\alpha/RT)$. Here α is the normalization constant, R is the gas constant and T is the temperature. Simulations were started at conditions corresponding to 500 K and then, to stabilize the knots in their ideal form, a computational annealing was performed by simulating an exponential decrease of the temperature of the system towards 0 K.

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were listed as distinct in knot tables compiled in 1899 by C. N. Little, and were believed by generations of topologists to correspond to different knot types until Perko demonstrated that they represent the same knot¹⁵. As starting configurations, we have taken Perko pair diagrams from the Rolfsen tables¹⁴ where they are denoted as knots 10_{161} and 10_{162} . The two final configurations obtained by our algorithm were practically identical so that we could overlap them. The ideal geometrical representation of the Perko knot is also shown in Fig. 1.

For each ideal form of the 21 types of knot analysed, we calculated the ratio of the axial length to the maximum diameter of the virtual tube centred on the axis of this ideal form (Table 1). The more complicated the knot, the greater is the length of flexible tube required to form such a knot. Using the length-to-diameter ratios of the ideal forms we can rank knots in terms of their complexity. By this criterion, for example, knot 5_1 is less complex than knot 5_2 and knot 8_{19} less complex than knot 8_1 .

We have also computed the writhe of the ideal forms of the knots (Table 1). The writhe is the average directional crossing number of the ideal form of the knot when viewed from all directions sampling the sphere equally and where left-handed crossings score as -1 and right-handed as $+1$ (ref. 16). Ideal representations of chiral knots showed very low writhe (for

TABLE 1 Characteristics of ideal geometrical representations of knots

Knot type	Axis length/ diameter	Writhe	Average crossing number
link 2_1	6.3	0.00	0.0
3_1	16.4	3.41	4.4
4_1	21.2	0.00	6.6
5_1	24.2	6.26	7.9
5_2	24.9	4.54	8.4
6_1	29.3	1.23	10.3
6_2	29.2	2.70	10.2
6_3	30.5	0.16	10.7
7_1	30.9	9.15	11.5
7_2	33.2	5.82	12.1
8_1	37.0	2.33	14.0
8_{18}	38.3	0.02	15.4
8_{19}	31.0	8.64	11.2
8_{20}	32.7	2.00	12.0
8_{21}	33.9	4.62	12.8
9_1	38.3	12.07	15.2
9_2	40.0	6.84	16.2
10_1	44.8	3.47	18.4
11_1	47.0	14.85	19.1
$3_1\#3_1$ (granny)	29.1	6.81	10.3
$3_1\#3_1^*$ (square)	28.9	0.01	9.6
Perko knot ($10_{161,162}$)	37.6	9.45	15.3

The length/diameter ratio was directly calculated for the ideal geometrical representation of a given type of knot. The writhe value¹⁶ was calculated using the gaussian integral formula $1/(4\pi) \oint_c \oint_c [(\mathbf{dr}_1 \times \mathbf{dr}_2) \cdot \mathbf{r}_{12}]/r_{12}^3$. To calculate the average crossing number a modified Gaussian integral formula was used, $1/(4\pi) \oint_c \oint_c [(\mathbf{dr}_1 \times \mathbf{dr}_2) \cdot \mathbf{r}_{12}]/r_{12}^3$. On integration, the vectors $\mathbf{r}_1$ and $\mathbf{r}_2$ run over the contour c , corresponding to the axial trajectory of the ideal geometrical representation of a given type of knot.

example, knot 6_3) or even adopted configurations with a writhe essentially equal to zero (for example, knot 4_1 and the square knot). Chiral knots have a substantial writhe (positive or negative depending on the actual chirality of the knot). Although the chiral knots analysed here have specific writhe values, these values do not correspond to the complexity of the knots but provide information about the extent of chirality. However, within a given family of knots, for example the torus-knots, the writhe is a good measure of the complexity. Figure 2a shows how the writhe of ideal representations of torus-knots (filled circles), and of twist-knots with even and odd numbers of crossings (filled squares and filled diamonds, respectively) increases linearly with the number of crossings that these knots have in standard tables. The writhe versus crossing-number relations are different for each family.

The average crossing number of the ideal representation (this number is always greater than the writhe as each left- and right-handed crossing score as $+1$) gives a good measure of knot complexity that is independent of knot type. In Table 1 we list the average crossing number for the knots we have analysed. When the average crossing numbers of the ideal forms of different knots are plotted against their length-to-diameter ratio (Table 1), a linear relationship is obtained that unifies many types of the analysed knots (Fig. 2b), including the composite knots square and granny, the non-alternating knots 8_{19} , 8_{20} and 8_{21} and the Perko knot.

The ideal representations of knots described here are also relevant to the behaviour of loosely knotted polymers undergoing thermal motion in good solvent conditions. Drawing on experience gained in modelling equilibrium configurations of DNA by the Metropolis Monte Carlo procedure^{12,17}, we decided to simulate millions of equilibrium configurations of nicked circular DNA molecules closed into different types of knots and to measure the writhe and average crossing number of each simulated configuration. (Although we simulated DNA chains, other polymers closed into knots should behave similarly.) We performed simulations for DNA molecules of 1,800 base pairs (bp) and 5,400 bp and considered only the situation in which the DNA is in good solvent

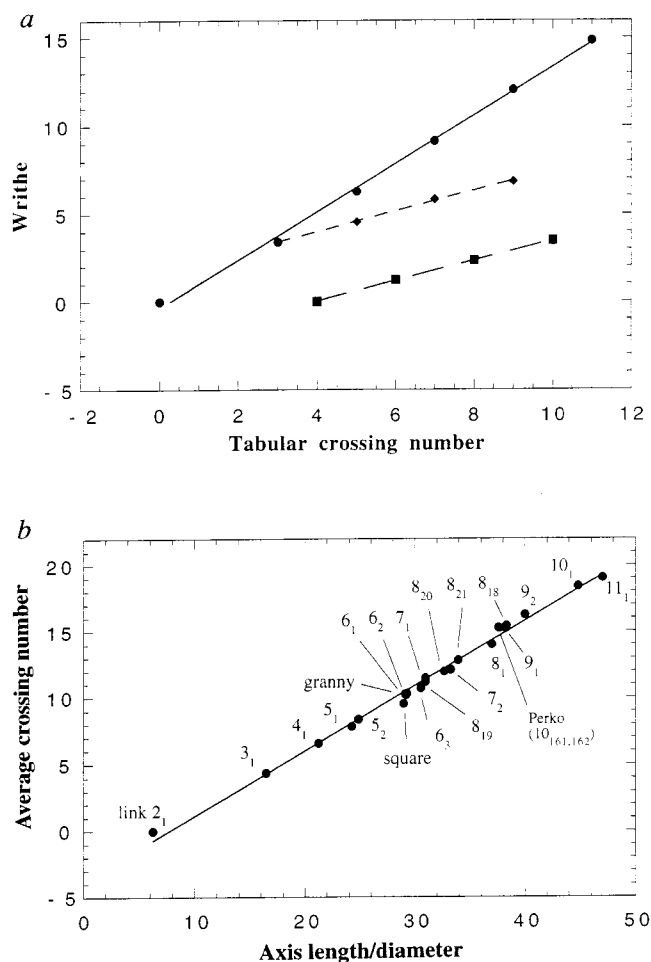


FIG. 2 Linear relations between ideal geometrical forms of different knots. a, Different families of knots show a linear increase of writhe with increasing standard crossing number. Filled circles, torus knots; filled diamonds and filled squares, twist knots with respectively odd and even numbers of crossings in their standard tabular representation. b, Length/diameter ratio and average crossing number of the ideal geometrical representations of different types of knots. Points corresponding to different knots are marked accordingly. For links 2_1 the values are given for one of the identical component rings.

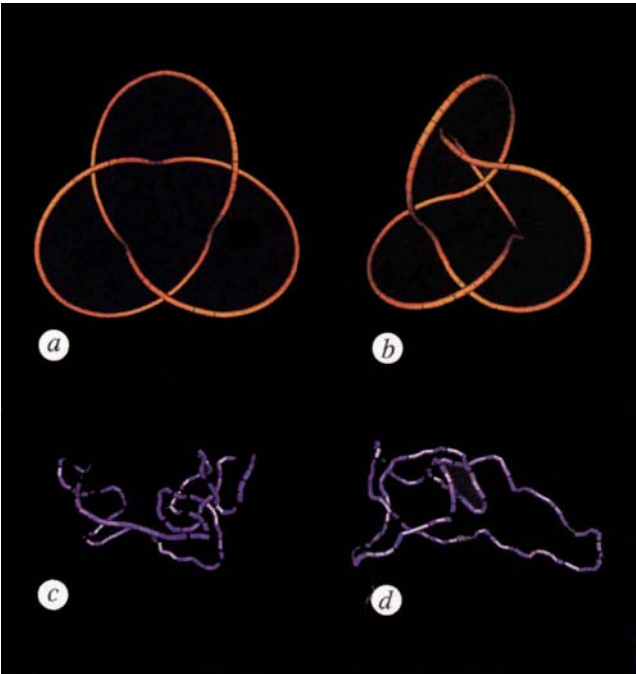


FIG. 3 Comparison between ideal trajectories of knots and of randomly distorted configurations: *a, b*, Ideal axial trajectories of trefoil (*a*) and four-noded (*b*) knot; *c, d*, corresponding configurations, simulated for a DNA molecule 5,400 bp long undergoing thermal motion.

TABLE 2 Characteristics of Boltzmann ensembles of knotted DNA chains

Knot type	5,400 bp		1,800 bp	
	(Writhe)	(Average crossing number)	(Writhe)	(Average crossing number)
link 2 ₁	0.00	10.6	0.00	3.13
3 ₁	3.43	14.6	3.41	6.72
4 ₁	0.01	17.2	0.00	8.80
5 ₁	6.30	18.7	6.21	10.1
5 ₂	4.56	19.2	4.59	10.5
6 ₁	1.20	21.1	1.18	12.2
6 ₂	2.83	21.4	2.76	12.4
6 ₃	0.04	21.7	0.01	12.8
7 ₁	9.10	22.5	8.95	13.5
7 ₂	5.79	23.0	5.77	14.1
8 ₁	2.47	24.9	2.43	16.1
9 ₁	12.0	26.4		
9 ₂	6.94	26.9		
10 ₁	3.60	28.6		

Mean writhe and mean crossing numbers of Boltzmann ensembles of thermally agitated DNA molecules closed into different types of knots. These values were obtained from Metropolis Monte Carlo simulations of equilibrium configurations of DNA molecules, 1,800-bp and 5,400-bp long, suspended in 40 mM NaCl. For shorter molecules (1,800 bp) we did not simulate knots with high knotting numbers as the entered effective diameter started interfering with equilibration of these knots.

conditions, that is, when there is no attraction between DNA segments. We set the DNA persistence length to 50 nm and its effective diameter to 9 nm, which would correspond to the moderate salt buffers used for gel electrophoresis¹⁸. The simulated molecules were treated as nicked, that is, there was no torsional stress. (For more details on the simulation procedure see ref. 11). Figure 3*c, d* shows randomly chosen equilibrium configurations of simulated trefoil and 4₁ knots tied on DNA molecules 5,400 bp long. It is obvious that the configurations shown have no resemblance to the axial paths of ideal configurations of the corresponding knots (Fig. 3*a, b*). But when the mean writhe values and the mean of the average crossing numbers, measured on millions of individual configurations within simulated Boltzmann ensembles of given types of knot were calculated (Table 2), we found a striking linear relation of these values to the corresponding values of ideal configuration of the analysed knots. The writhe values for different types of knots seem to be independent of DNA size and are equal (within the statistical error range) to the writhe values of the ideal forms of the corresponding knots (Fig. 4*a*). The mean crossing number for Boltzmann ensembles of simulated configurations increases with the size of the molecule (compare the values for DNA chains 1,800 and 5,400 bp long). However, for a given size of simulated chain, there is an almost perfect linear relation between the average crossing number of ensembles of simulated trajectories and the average crossing number of ideal configurations of the knot types tested. We note that irrespective of the size of simulated chains the slope of correlation is close to 1. Longer knotted chains just have more additional random crossings than shorter chains, so the number of additional crossings is practically independent of the type of knot (Fig. 4*b*).

We recall Plato's distinction between ideal and real objects.

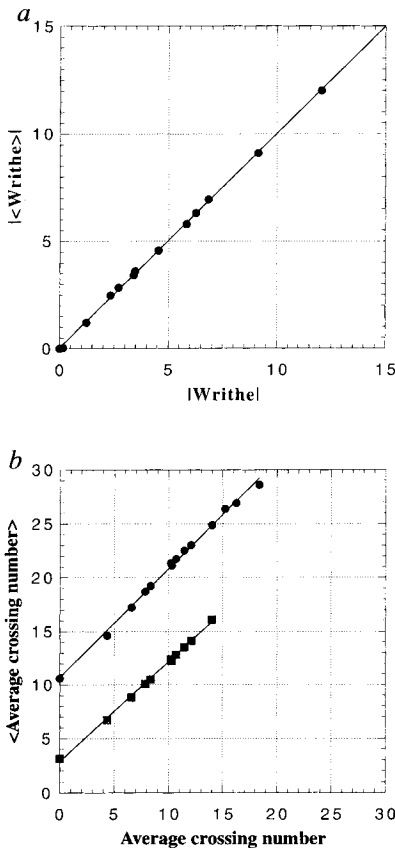


FIG. 4 Relations between ideal configurations of knots and knotted DNA chains undergoing thermal motion. *a*, 1:1 correspondence between writhe of ideal forms of knots and mean writhe value of ensembles of $\sim 9 \times 10^6$ configurations simulating behaviour of knotted double-stranded DNA molecules (5,400 bp long) randomly distorted by thermal motion. We note that each point on the graph corresponds to a different knot type. *b*, Linear correlation between average crossing number of ideal configurations and the mean of the average crossing numbers of ensembles of simulated configurations of knotted DNA chains with 1,800 (filled squares) and 5,400 base pairs (filled circles).

Plato proposed that, although real objects cannot have all the properties of ideal geometrical objects, certain reflections of ideality should still be present in real objects. Knotted polymeric chains provide an interesting example of a relation between ideal geometrical representations and real physical objects. □

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Dislocation-mediated melting of a two-dimensional crystal

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MELTING of three-dimensional solids usually starts at the free surface, which typically melts at a lower temperature than the bulk material¹. In two dimensions the starting point of many studies is the Kosterlitz–Thouless theory^{2,3}, in which melting is initiated through dislocation unbinding. Langmuir monolayers—single layers of amphiphilic molecules formed at the air–water interface—should provide an ideal model for studying melting in two dimensions. Here we show that for monolayer crystals of fatty acids coexisting with their liquid phase, the interior melts before the edges. The melting of crystals under mechanical stress is initiated along the line at which the internal stress vanishes. We suggest that this apparently counterintuitive result arises from defect migration to the region of zero stress, where they accumulate and nucleate melting. These results support the idea that defects play a crucial role in melting of two-dimensional systems.

We prepared two-dimensional crystals by compression of a Langmuir film of pure NBD-stearic acid^{4–6} (12-(*N*-methyl)-*N*-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)octadecanoic acid). Rectangular crystals (typically $40 \times 1,000 \mu\text{m}$) nucleate and grow in the two-dimensional liquid phase with which they are in equilibrium. The crystallization transition is strongly first-order: the area per molecule in the liquid phase is three times larger than in the solid phase^{4,5}. Moreover, the Young's modulus E of these crystals is 6 ± 2 times the value calculated at the dislocation unbinding transition⁶. Melting of the crystals can be achieved by two different thermodynamic processes (heating and decompression) and by a photochemical process. In the latter process,

illumination with blue light (which is strongly absorbed by the crystals) shifts the solid/liquid phase boundary, leading to the melting of the crystal. This occurs through a reversible photochemical reaction with oxygen in the air leading to freezing-point depression. The three processes lead to strictly analogous behaviour, indicating a common mechanism for the initiation of the melting. The melting starts either from the short sides of the crystal or from the inside. The dynamics of melting are very slow and the long sides of the crystal are very difficult to melt, leaving two strips of crystal each $2 \mu\text{m}$ wide after the bulk has melted completely (Fig. 1). For melting by a heating, a period of ~ 120 min is necessary to melt the bulk for a temperature increase of 1°C (30 min for 4°C) above the equilibrium temperature. On this timescale, the remaining strips melt only after an increase in temperature of $\sim 10^\circ\text{C}$. Melting obtained by decreasing the surface pressure (Fig. 1a, b) or by illumination (Fig. 1d) shows identical behaviour. The $2 \mu\text{m}$ length scale is reproducible from experiment to experiment and is independent of the width of the crystal.

Two new boundaries can be created by breaking the two-dimensional crystal into two pieces along a line roughly parallel to the long sides (Fig. 2a). If these pieces of crystal are illuminated just after their creation, the two newly created boundaries and the bulk melt simultaneously. When illuminated more than 20 minutes after the breaking, the two new boundaries do not melt (Fig. 2b). During this waiting time, the width of the pieces of crystal is

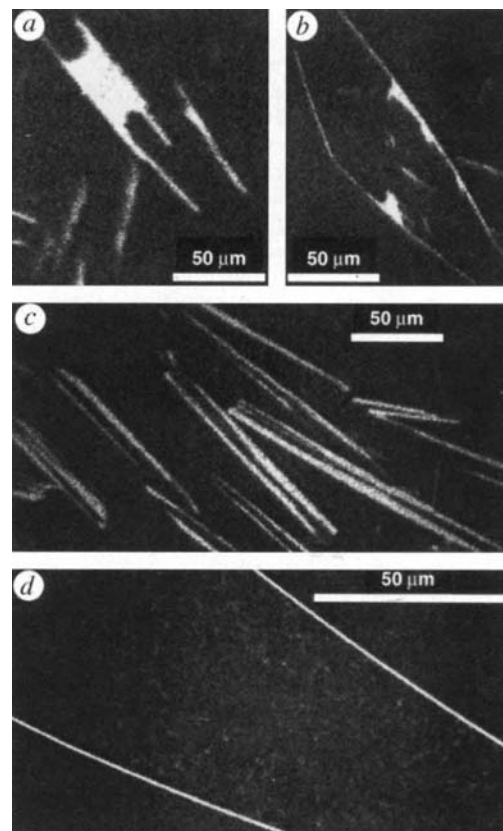


FIG. 1 Fluorescence microscopy images of the NBD-stearic acid system at solid–liquid phase coexistence. In the two-dimensional liquid phase the fluorescence is quenched, whereas the solid domains fluoresce and appear light against a dark background. a, Melting obtained by decompression of the rectangular crystals; it starts from the two short sides. In b, two strips (one along each long side) resist melting. c, Melting obtained by heating; the image was taken 120 min after a temperature increase of 1°C . d, Melting obtained by illumination. The melting occurs everywhere inside the crystal while the strip along each long side does not melt.

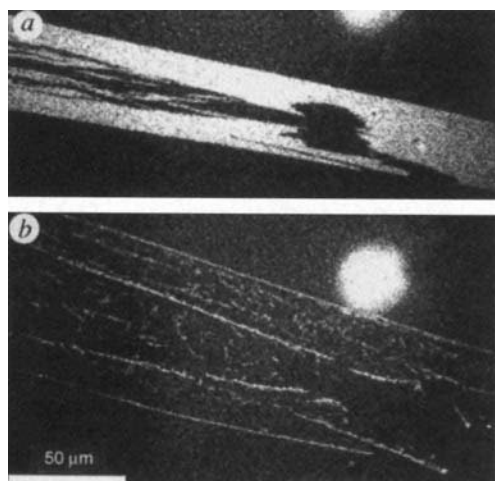


FIG. 2 Breaking and subsequent melting of a two-dimensional crystal. *a*, A small area within the crystal is illuminated until it melts. The increase of surface area upon melting (due to the large difference in area per molecule between the solid and the liquid phase) induces a crack approximately parallel to the long sides of the crystal (because the mechanical properties are anisotropic), creating two new boundaries. *b*, Melting by illumination of the broken crystal 20 min after the breaking. The boundaries along the two long sides of the crystal and the two newly created boundaries do not melt. The bright spot is one of the glass fibres used to immobilize the crystal.

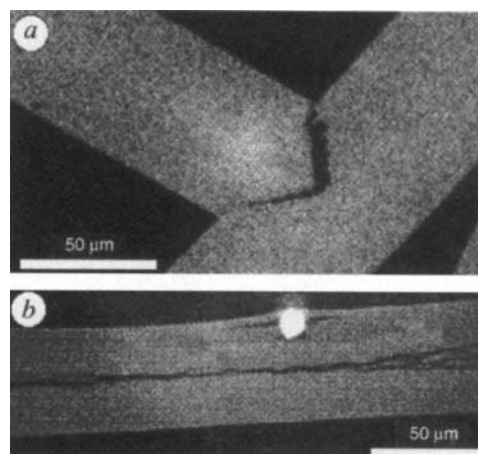


FIG. 3 Local melting by illumination of a crystal. *a*, Rapid melting of a grain boundary between two crystals which were together before illumination. *b*, Melting of a crystal under a bending stress. The melting appears along the neutral line. The bright spot is the image of one of the glass fibres used to bend the crystal. (Two glass fibres immobilize the long ends of the crystal, while a third, mobile, fibre that is apparent on the image is used to apply a force perpendicular to the long axis of the crystal, in its middle. See ref. 6 for details). This fibre has partially penetrated the crystal, inducing a local crack in its vicinity.

constant: the new strips that resist melting result from a change with time of the crystal border. These observations suggest that either the two strips are a different phase, due to a boundary effect, or that the melting nucleates around defects that are expelled from these strips. Order induced by surface effects has been observed in three-dimensional liquid crystals⁷, but on length scales much smaller than $2\ \mu\text{m}$. The long-range dipolar forces that exist in two-dimensional films could explain such long-range effects in our case. However, looking at the polarization of the fluorescence, no difference between the border and the volume can be detected. Also, electron-diffraction experiments⁸ repeatedly show only one diffraction pattern. Although the strips along the borders were not specifically looked at in this last experiment, the solid appears to be a single crystal.

The important role of the defects in the initiation of the melting process is revealed by two observations. First, grain boundaries melt much more quickly than the inside of a single crystal (after respectively 5 s and 1 min of illumination under the microscope; Fig. 3*a*). As a grain boundary is an array of dislocations⁹, this result indicates that the melting nucleates around defects. A second and very striking observation is that melting by illumination of a two-dimensional crystal under a bending stress (applied with three glass fibres⁶) starts along the neutral line (Fig. 3*b*) where the stress vanishes. From thermodynamics, one expects the contrary to happen: as the stress is released by melting the crystal, the nucleation of the liquid phase should be easier at the points of maximum stress¹⁰. Surprisingly, melting along the neutral line is also observed when the crystal is illuminated a short time after the stress is relaxed: the crystal has a memory of the stress. However, if we wait for a time $\tau_m \approx 180\ \text{s}$ after the stress release, the crystal melts as if the stress had never been applied. This suggests that melting nucleates around defects that migrate very quickly through the crystal with a diffusion coefficient $D \approx d^2/4\tau_m$, where d is the width of the crystal. This yields $D \approx 10^{-8}\ \text{cm}^2\ \text{s}^{-1}$, which may be compared with an estimate for dislocation glide ($D \approx 10^{-6}\ \text{cm}^2\ \text{s}^{-1}$) or dislocation climb ($D \approx 10^{-10}\ \text{cm}^2\ \text{s}^{-1}$) for two-dimensional crystals⁹. In the stress field, the Peach–Koehler force¹¹ ($f_p(y) = \sigma b$) causes the dislocations to move ($b = 15 \times 10^{-10}\ \text{m}$ is the Burger vector and $\sigma = Ey/R$ is the stress at a distance y from the neutral line in a bent crystal with

radius of curvature R). Depending on their Burgers vector and on the stress field under consideration, the dislocations are advected either to the neutral line or to the crystal border. Under our experimental conditions, the energy of the dislocations advected to the neutral line $\int_0^y f_p(u)du$ is large compared to the thermal energy, $k_B T$, for $y \gtrsim 1\ \mu\text{m}$; thus they are rapidly accumulated very close to this line.

Our results imply that, in the absence of external stress, defects must be removed from the crystal edges. This is consistent with fracture experiments on this system¹², in which a delay before breaking was attributed to the spontaneous nucleation of cracks, suggesting that there are no defects at the boundaries. Near the boundary, dislocations interact attractively with their image dislocations (which express mathematically the interaction with the boundary) and so can be removed from the boundary region. However, the large value of E opposes this interaction. Consequently, the large length scale of $2\ \mu\text{m}$ remains a puzzle.

Experiments on melting in three dimensions reveal that superheating of a solid is possible, provided that the liquid phase does not completely wet the solid–gas interface: there is an energy cost for creating the liquid phase bounded by two new interfaces. The main difference from our experiment is that the two-dimensional solid already coexists with its liquid phase. Consequently, line tension effects should not be important for the ‘surface melting’ of the two-dimensional crystal, as no new line interfaces are created. Together with the observed slowness of the melting of two-dimensional crystals of NBD-stearic acid (but also found for other compounds giving very rigid two-dimensional crystals), this implies a large energetic barrier for the formation of the liquid phase. $\square$

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Nanotubes as nanoprobe in scanning probe microscopy

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SINCE the invention of the scanning tunnelling microscope¹, the value of establishing a physical connection between the macroscopic world and individual nanometre-scale objects has become increasingly evident, both for probing these objects^{2–4} and for direct manipulation^{5–7} and fabrication^{8–10} at the nanometre scale. While good progress has been made in controlling the position of the macroscopic probe of such devices to sub-ångström accuracy, and in designing sensitive detection schemes, less has been done to improve the probe tip itself⁴. Ideally the tip should be as precisely defined as the object under investigation, and should maintain its integrity after repeated use not only in high vacuum but also in air and water. The best tips currently used for scanning probe microscopy do sometimes achieve sub-nanometre resolution, but they seldom survive a ‘tip crash’ with the surface, and it is rarely clear what the atomic configuration of the tip is during imaging. Here we show that carbon nanotubes^{11,12} might constitute well defined tips for scanning probe microscopy. We have attached individual nanotubes several micrometres in length to the silicon cantilevers of conventional atomic force microscopes. Because of their flexibility, the tips are resistant to damage from tip crashes, while their slenderness permits imaging of sharp recesses in surface topography. We have also been able to exploit the electrical conductivity of nanotubes by using them for scanning tunnelling microscopy.

Multiwalled nanotubes (MWNTs) were prepared in the optimized direct-current carbon arc apparatus reported previously¹³. To use a nanotube as a robust probe we bonded it to the side of the tip of a conventional silicon cantilever using a soft acrylic adhesive 1–10 nm thick (Fig. 1). This permits the nanotube to bend away from its connection whenever the tip is inadvertently ‘crashed’ into a hard surface, and then to snap back to its original straight position when the tip is withdrawn. Effectively the nanotube is then ‘spring loaded’ much like the side-view mirror of a car.

When used in tapping-mode scanning force microscopy, SFM (where the change in amplitude of an oscillating cantilever driven near its resonant frequency is monitored as the tip taps the surface; the sharp frequency response of high-quality cantilevers make this technique exquisitely sensitive), a carbon nanotube tip such as that shown in Fig. 1 has the unusual advantage that it is both stiff and gentle. It is stiff because there is no bending of the nanotube at all when it encounters a surface at near-normal incidence until the Euler buckling force¹⁴, F_{EULER} is exceeded:

$$F_{\text{EULER}} = \pi^2 YI/L^2 \quad (1)$$

where Y is the Young’s modulus, I is the stress moment over the cross-section of the nanotube of radius r ($I \approx \pi r^4/4$) and L is the

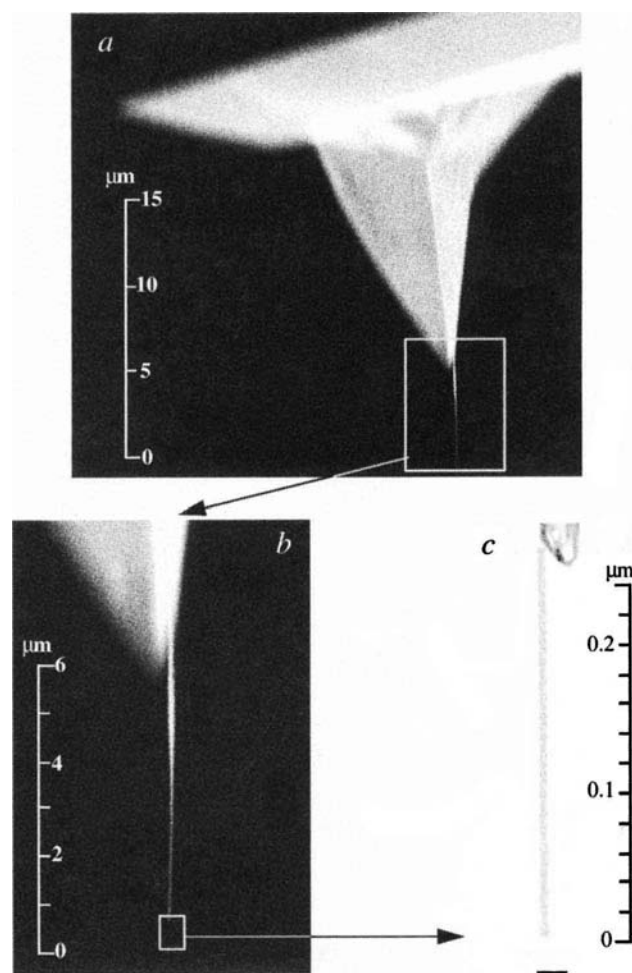


FIG. 1 Single nanotube attached to the pyramidal tip of a silicon cantilever for scanning force microscopy (SFM). The nanotube was attached by first coating the bottom 1–2 μm section of the silicon tip with an acrylic adhesive (by sticking it slightly into an adhesive-coated carbon tape (Electron Microscopy Services, Fort Washington, PA)), and then bringing this tip into contact with the side of a bundle of 5–10 multiwalled carbon nanotubes (MWNTs) while under direct view of an optical microscope using dark-field illumination. Once attached, the nanotube bundle was pulled free from its connections with other nanotubes, leaving a single 5-nm-diameter MWNT extending along the final 250 nm. (The most common MWNT diameter obtained at the tip is 5–20 nm, and lengths of single MWNT up to 1 μm are capable of good imaging.) Higher-resolution transmission electron microscopy (TEM) images of this tube showed it to be closed at the tip, as is typical for most MWNTs produced by this arc method (without oxidative etching). *a*, *b*, Scanning electron microscope (SEM) images showing the MWNT bundle attached to the steeper slope of the back side of the silicon pyramid. This attachment ensured that the individual MWNT extending out the end of the bundle, as seen in the TEM image *c*, would approach the surface to be imaged within a few degrees of vertical. Although the adhesive bonding is restricted to within 1–2 μm of the apex, the MWNT bundle continues 5–10 μm further along the side of the pyramid in van der Waals contact. If the pyramid itself is pre-coated with a layer of conductive metal, this method produces a good electrical contact to the MWNT bundle and thereby to the single MWNT probe tip at the end²⁵. This attachment method is simple and reliable once the requisite microscope and micro-manipulators are set up. With good-quality MWNT material, mounted probes similar to that shown can be obtained after a few tries. It is useful to have ready access to an electron microscope with a specially-adapted specimen holder to view the cantilever after the MWNT bundle has been attached to see whether the final MWNT at the tip is of the length and diameter desired; after some experience, however, one can simply try imaging with the tips and electro-shortening if necessary. Scale bar in *c*, 20 nm.

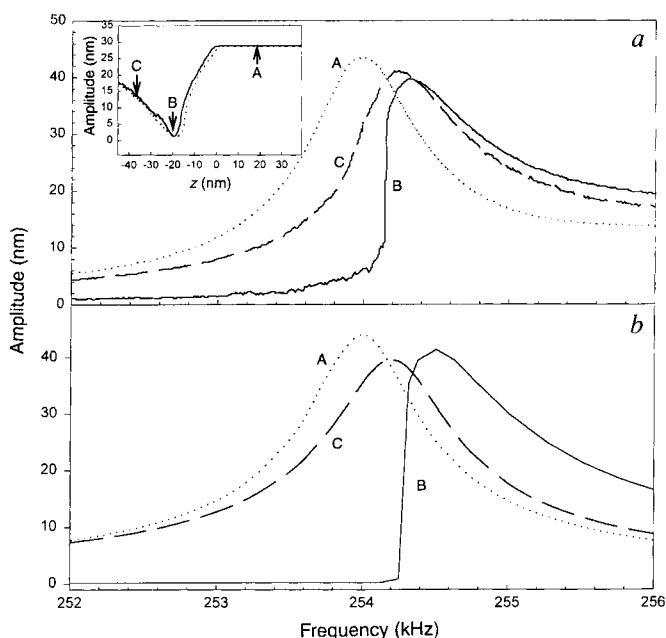
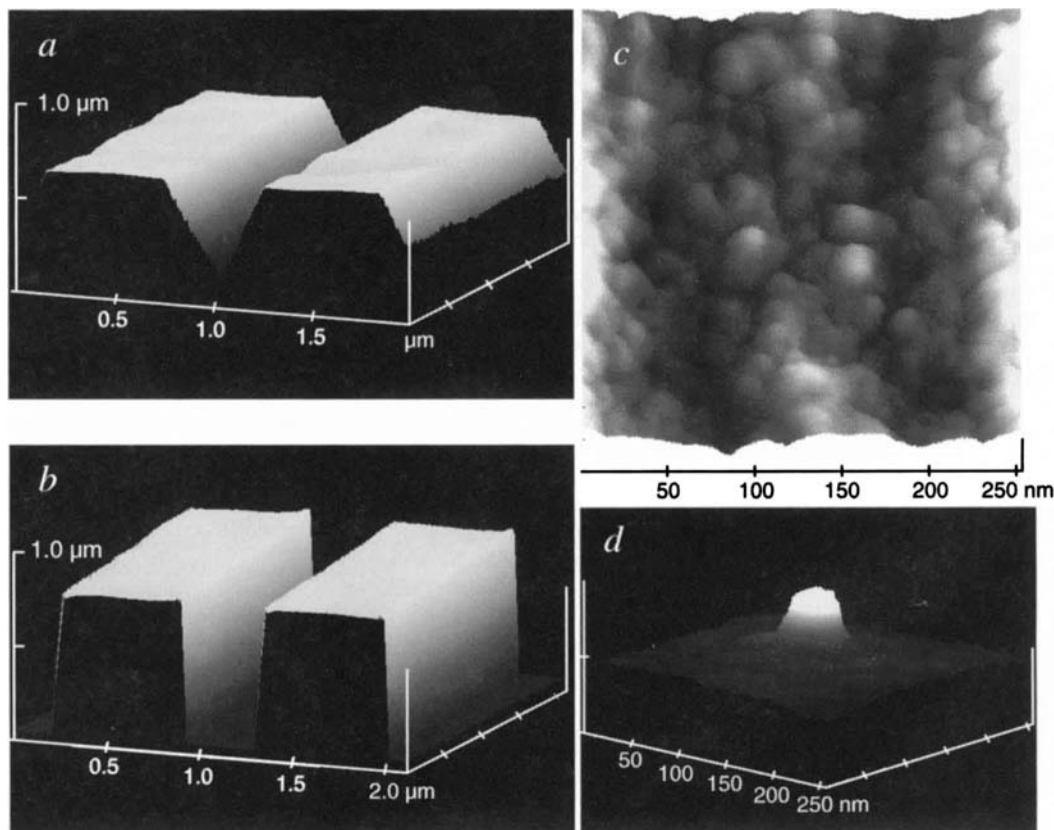


FIG. 2 Frequency response of cantilevers with mounted nanotube tips. The inset of panel *a* shows the tapping amplitude change as a function of distance (z) between a freshly cleaved mica surface and the tip of the nanotube (shown in Fig. 1). *a*, Three frequency scans were taken at the surface heights marked on the inset: A while the cantilever was oscillating freely in air, B while the nanotube at the tip of the cantilever was hitting the surface at the midpoint of the oscillation, and C while the nanotube was flexing in continuous contact with the surface throughout the oscillation. *b*, The result of a direct numerical simulation of the experiment of *a*, assuming the equation of motion of the cantilever is $Md^2z/dt^2 = F - P\sin(\omega t - \phi)$, where frequency $F = F_{ar} = k_c z + C dz/dt$ when the cantilever is oscillating freely in air, and $F = F_{bit} + F_{EULER} + C_n dz/dt$ when the nanotube is in contact with the surface, P is the amplitude of the driving oscillator at frequency ω , $C = M\omega_c/Q_c$ is the viscous damping factor of the cantilever alone, $C_n = M\omega_c/Q_n$ is an additional damping term due to flexing of the nanotube, M is the effective mass of the cantilever (6×10^{-6} g), and F_{EULER} is the Euler buckling force (equation (1)). (Here ϕ is a phase shift, k_c is the cantilever force constant, and Q_c and Q_n are the quality factors of the cantilever and nanotube, respectively). The simulation plotted in *b* is for $\omega_c/2\pi = 234.8$ kHz, $k_n = 100$ N m $^{-1}$, $Q_c = 360$, $Q_n = 3,000$, P adjusted to give a peak-to-peak amplitude in air of 44 nm, and $F_{EULER} = 10$ nN $[L_0/(L_0 - (Z_s - z))]^2$, where $L_0 = 250$ nm, and Z_s is the z position of the mica surface as marked on the inset to *a*. The sharpness of the recovery of oscillation amplitude near $\omega^* = 254.2$ kHz was found to be a sensitive function of the buckling force, with the best fit to the data requiring 8 nN $< F_{EULER} < 10$ nN, consistent with the expectation^{26,27} that the Young's modulus of a MWNT be similar to that of in-plane graphite. In contrast to conventional tips which are often damaged by force and amplitude versus distance scans such as shown in the inset to *a*, the carbon nanotube tips are found to be extremely robust—even surviving repeated hard 'crashes' which bend the nanotube entirely out of the way, enabling the pyramidal silicon tip itself to press onto the surface.

FIG. 3 Tapping-mode SFM image of a 400-nm-wide, 800-nm-deep trench etched through a TiN-coated aluminum film on a silicon wafer. *a*, Image taken with a bare pyramidal silicon tip (Digital Instruments TESP cantilever, 250 kHz resonant frequency, 100 N m $^{-1}$ force constant). The apparent triangular shape of the trench is an artefact due to the pyramidal shape of the imaging tip. *b*, Image taken with a nanotube attached to the pyramid of this same TESP cantilever much like that shown in Fig. 1. This thin, long nanotube is now able to reach to the bottom of the trench as shown in detail in *c* where the texture of the bottom is clearly imaged, revealing that the mean surface roughness at the bottom of the trench is ± 0.4 nm. Nanotube probes provided equal or better resolution than commercial tips in all of the samples examined so far (five or six various types). *d*, Tapping-mode SFM image of a 40-nm-diameter 30-nm-high carbon dot formed on the bottom of the trench by applying a -5 V voltage pulse to the nanotube while it was held 30 nm above the bottom, after applying a 5-nm-thick gold coating to render the surface of the trench electrically conducting. Note that the axis numbers in *a* and *b* are μ m, and those in *c* and *d* are nm.



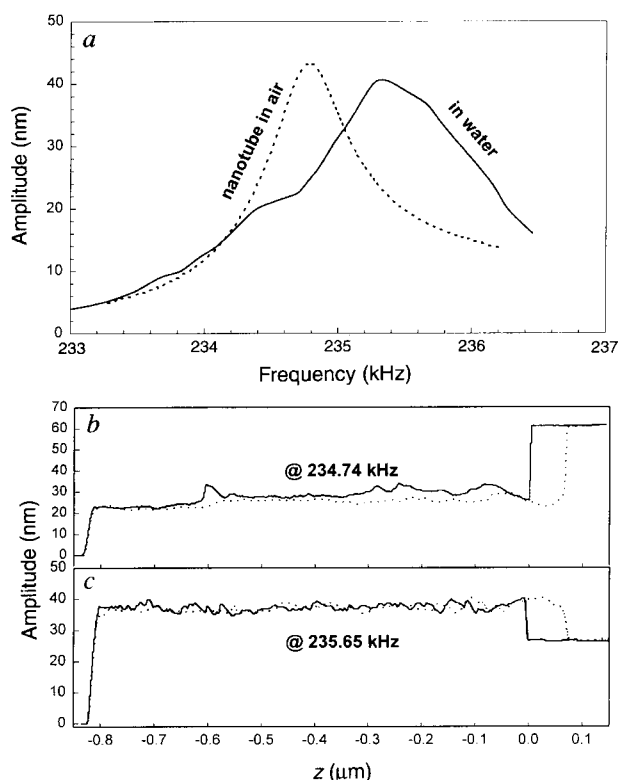


FIG. 4 **a**, Frequency response of a cantilever in air (dashed line), and with the nanotube at its tip extending 0.8 μm into a water-flooded trench similar to that shown in Fig. 3 (solid line), illustrating that such nanotube tips will permit tapping-mode experiments to be done under water even though the cantilever itself remains entirely in air. Amplitude change on dipping a nanotube probe into the flooded trench. The first contact with the water surface occurs at $z = 0$. **b**, **c**, The nanotube tip encounters the bottom of the trench at $z = -820$ nm. The trace in **b** was done at the resonant frequency of the cantilever oscillating in air (234.74 kHz); the trace in **c** was obtained at 235.65 kHz where the oscillation amplitude is seen to substantially increase when the tip of the nanotube penetrates the water surface. Although we have obtained images under these conditions, they are not yet up to the standard of those in air, for reasons we believe have to do with deficiencies in our current fluid cell. We are currently working on improving the situation, and are confident that ultimately we will obtain very high-quality images under water.

length of the nanotube. Assuming the Young's modulus of the nanotube is similar to in-plane graphite (~ 1 TPa), the Euler buckling force of the 250-nm-long 5-nm-diameter MWNT tip shown mounted in Fig. 1 is ~ 5 nN. But the nanotube is also gentle because once the buckling force is exceeded the nanotube will bend easily through large amplitudes with little additional force. Euler buckling therefore serves as a kind of insurance policy during SFM imaging: the maximum force that can be transmitted to the sample is F_{EULER} . In addition, the MWNT tip is extremely gentle when touching an object laterally. The bending motion for side-directed forces is harmonic with a force constant $k_n = 3YI/L^3$. For the MWNT tip of Fig. 1, $k_n = 6.3$ pN nm $^{-1}$. When used in tapping mode, the MWNT tip shown in Fig. 1 behaves in accord with these expectations, as demonstrated in Fig. 2.

The mechanism for reduction in the tapping amplitude in operation here is almost entirely elastic. The spring force from the bending nanotube produces a coherent de-excitation of the cantilever oscillation at driving frequencies below the critical frequency ω^* . The result is that gentle, reliable SFM imaging may be accomplished in the tapping mode with even extremely stiff, high-resonant-frequency cantilevers. In contrast to the hard

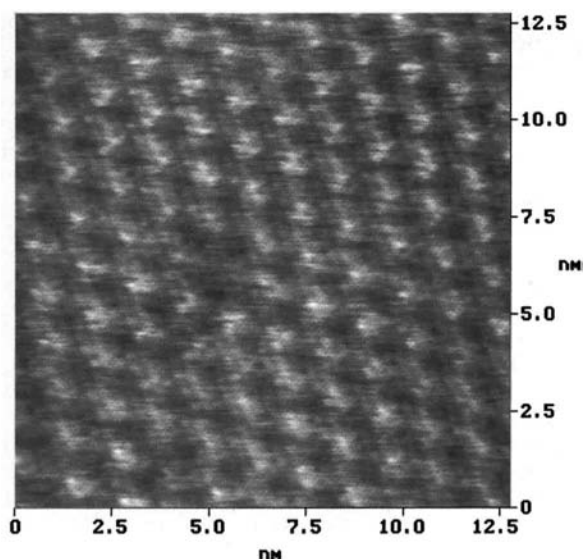


FIG. 5 Unfiltered constant-height STM image exhibiting atomic resolution and charge density waves on freshly cleaved TaS_2 surface, performed with a MWNT tip mounted similarly to that shown in Fig. 1. Bias voltage 16 mV, tunnelling current 810 pA.

silicon pyramidal tip, which can easily generate impact forces >100 nN per tap if the scan is not carefully controlled¹⁵, the MWNT probe serves as a compliant spring which moderates the impact of each tap on the surface, the peak force never exceeding F_{EULER} .

Figure 3 shows that these long, narrow MWNT tips can reach into deep trenches previously inaccessible to high-resolution scanning probes. The normal pyramidal tip is simply too wide to reach the bottom of a 0.4 μm wide 0.8 μm deep trench (Fig. 3a), whereas the nanotube permits the roughness of the silica surface at the bottom to be imaged easily. Also, as shown in Fig. 3d, it is possible using a voltage pulse on the nanotube to deposit a 40-nm dot of carbon at the bottom of the trench, and then to go back and image. Owing to the 'spring loading' of the MWNT bundle to the cantilever and the high strength and flexibility of the carbon nanotubes, SFM imaging of tortuous structures such as the trenches shown in Fig. 3 can be done without fear of damage either to the MWNT tip or the trench structure itself. In contrast, we were unable to image these trenches with an Ultralever (Park Scientific Instruments, Sunnyvale, CA) having a 4- μm -long conical silicon tip. After a few minutes of admittedly rather clumsy scanning, 80 nm of the conical tip had been broken off—a problem we expect will plague any sharp, brittle nanostructure rigidly attached at the tip of a scanning probe.

One of the principal limits in SFM imaging in air has been that at normal humidity the surface is covered with a layer of water, and the capillary adhesion forces produced when the tip makes contact are typically 10–100 nN (ref. 16). As a result, one is forced to use high-force-constant cantilevers oscillating with substantial amplitude to ensure that the tip does not get caught by the surface. Owing to the small diameter of the nanotube, we find the capillary adhesion force of MWNT tips is generally reduced to <5 nN and often as low as 0.05 nN, permitting tapping-mode imaging with cantilevers having force constants as small as 0.01 N m $^{-1}$ at a peak-to-peak amplitude of 10 nm.

To get away entirely from the capillary adhesion force it is now conventional to image under some fluid¹⁷—normally water⁴. However, now that the cantilever must oscillate in water it is no longer possible to operate at high frequency and high Q (ref. 18). With a MWNT tip like the one shown in Fig. 1, it is now possible to immerse only the nanotube under the water, leaving the cantilever

free to oscillate in air. We have tested the feasibility of this idea by flooding the trenches seen in Fig. 3 with water. Figure 4 shows that the frequency dependence of the cantilever oscillation is only slightly affected when the lower 0.7 μm length of the nanotube is immersed in water within the trench. Also shown in Fig. 4 is the amplitude of the cantilever oscillation as a function of distance from the meniscus at the top of the trench. Note that for the bottom trace which was taken at 235.65 kHz (0.85 kHz above the resonant frequency of the cantilever in air) there is actually an increase in the oscillation amplitude when the MWNT is in the water. We suspect that this effect is due to the elasticity of the meniscus formed around the nanotube at the top of the water layer. Note also that the oscillation amplitude drops sharply when the MWNT tip encounters the SiO_2 surface at the bottom of the trench, permitting SFM imaging of this surface under water at the same frequency and with nearly the same Q obtained in air.

Because the MWNTs are electrically conductive^{19,20} they may be used as probes for scanning tunnelling microscopy, STM, and in various scanning electrochemical modes^{21,22} as well. Figure 5 shows an example of atomic-scale-resolution STM using a carbon nanotube to image the charge density waves on a freshly cleaved 1T-TaS₂ surface²³.

We have been successful as well in attaching a single 'rope' of single-walled nanotube (SWNT) material²⁴ to the side of a MWNT probe tip similar to the one shown in Fig. 1. As with the MWNT tip itself, we find it is possible to adjust, at least crudely (± 20 nm), the length of the SWNT rope extending out from the tip by applying a voltage pulse that generates a momentary nanoscale arc between the tip and a conductive surface 10–50 nm away. However, to realize the full promise these nanotubes hold as probes of the nanometre world, a more precise method will be needed: one that will guarantee, for example, that just a single (10,10) nanotube²⁴ extends out the desired length from a bundle of other nanotubes. Laser annealing of the tip of such a single (10,10) tube should ensure that the tip of this SWNT nanoprobe is perfectly closed with a C_{5v} symmetry hemifullerene dome, having a single pentagon centred on the five-fold symmetry axis at the vertex. Used either bare or with chemical derivatization at specific sites on the tip, such a (10,10) tube would be a good candidate for the ultimate nanoprobe. $\square$

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Self-replicating amphiphilic monolayers

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MOLECULAR films with predetermined layered structures can be engineered by using techniques such as the Langmuir–Blodgett method^{1,2} and self-assembly^{3–9} to deposit discrete monolayers sequentially on a substrate. Such films might have a variety of uses—as smart surface coatings, nonlinear optical materials and in tribology, for example. Here we report the replicative growth of a molecular film of self-assembling silane bilayers with hydrogen-interlayer polar regions into which further identical bilayers can be intercalated. The intercalation step is triggered by a chemical treatment and so can be carried out controllably, allowing duplication in one step of an entire multilayer structure. In this way, we can achieve the stepwise exponential growth of a multilayer film with a predictable number of stacked bilayers.

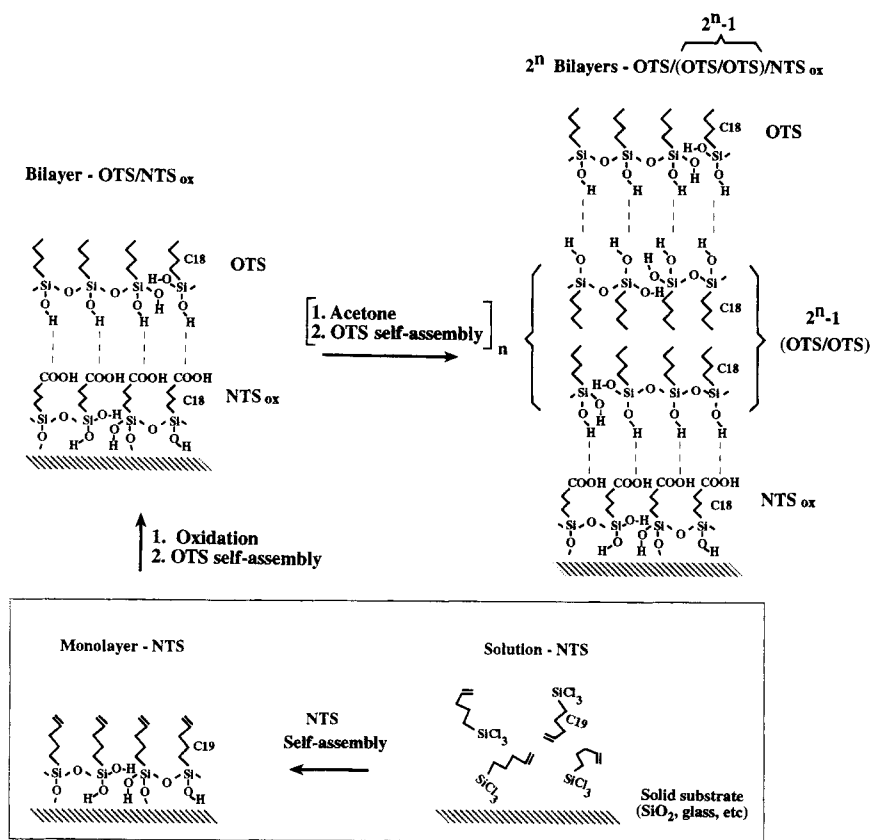
The quest for artificial molecular entities endowed with the ability to self-replicate in a controllable manner provides much of the stimulus for current research in supramolecular chemistry^{10–18}. Particular attention has been given to the study of molecular self-assembly, recognition and organization on a template—elements commonly regarded as prerequisites for the emergence of self-replication in a molecular system. Our recent studies on layer-by-layer self-assembled silane multilayers with interlayer hydrogen bonding^{19,20} led us to examine the possibility of using the facile intercalation and template-induced organization in such systems as a means for achieving self-replication.

Here we report on the replicative growth of a solid-immobilized multilayer of oriented long-chain silanes (Fig. 1). We start with an initial hydrogen-bonded bilayer prepared by sequential monolayer self-assembly from solution²⁰, and proceed through two consecutive steps that involve the acetone-mediated insertion of water into the silane–carboxylic acid interlayer polar space of the pre-assembled bilayer, followed by the spontaneous intercalation of a new silane bilayer (OTS/OTS) into the acetone-treated initial bilayer, upon its immersion in a solution of OTS in bicyclohexyl (BCH; OTS is *n*-octadecyltrichlorosilane.) By repeating this two-step procedure, we observed the repeated deposition of similarly structured OTS/OTS bilayers, the number of fresh bilayers added to the film on completion of each such sequence of operations being equal to the total number of intercalable bilayers already present on the surface. By repeatedly duplicating the entire set of predeposited bilayers, the total number of such bilayers generated on the surface grows as $2^n - 1$, where n is the number of acetone–OTS treatment cycles (Fig. 1).

The stepwise exponential growth of ordered multilayer films, according to the sequence of operations outlined above, is demonstrated by Fourier transform infrared (FTIR) spectra taken before and after each film deposition operation. The FTIR data provide direct evidence for the total amount of film-forming material (OTS) deposited on the surface^{19,20}, and for the orientation and packing density of the OTS paraffinic tails^{20–23} in each deposited layer and the chemical transformations (hydrolysis, condensation) affecting the silane head groups on their incorporation into the growing surface film^{20,24,25}.

For example, the H–C–H stretch bands (at 2,917 and 2,850 cm^{-1}) of the normalized spectral curves in Fig. 2—representing single NTS and OTS monolayers, the initial bilayer, and thicker films (between 8 and 32 stacked monolayers) produced by the present process—are practically superimposable. (Here NTS

FIG. 1 Schematic representation of the replicative growth of a solid-immobilized multilayer film of oriented long-chain silanes involving the externally triggered duplication of individual bilayers. The initial OTS/NTS_{ox} bilayer (top left) was prepared by the self-assembly of the precursor NTS (nonadecenyltrichlorosilane, CH₂=CH-(CH₂)₁₇-SiCl₃) (inset) and OTS (*n*-octadecyltrichlorosilane, CH₃-(CH₂)₁₇-SiCl₃) monolayers from 5.0 × 10⁻³ M solutions of the respective silanes in pure BCH (0.5–1.0 min immersion times, ambient temperature)²⁰, with the NTS terminal double bonds being oxidized to -COOH (left) by the KMnO₄-crown ether (dicyclohexano-18-crown 6) complex in benzene^{3,20}. Each acetone-OTS treatment cycle (top) consisted of exposure of the film to analytical grade acetone under reflux in a Soxhlet extractor for ~5 min, followed by a 30–50 min pause during which infrared measurements were performed on some of the film samples, then a second identical 5 min acetone treatment, immediately followed by immersion in the OTS solution in BCH (5.0 × 10⁻³ M) for ~1 min (from which the film emerges completely dry), and final sonication in clean decaline for ~2 min, in order to remove microdroplets of the OTS solution that may still adhere to the surface or edges of the film-coated substrate. The final film is shown to contain 2ⁿ - 1 OTS/OTS stacked bilayers (top right), *n* being the number of acetone-OTS treatment cycles. For clarity, only 3 out of the 17 chain methylenes of each of the film components, NTS (C19), NTS_{ox} (C18) and OTS (C18), are indicated. To account for the dynamic equilibration of the Si-O linkages in hydrogen-bonded silane multilayers²⁰, the silane polar regions are drawn as randomly distributed siloxanes (-Si-O-Si-) and silanols (-Si-OH), only limited covalent bonding being established even between the first monolayer and the underlying solid substrate.

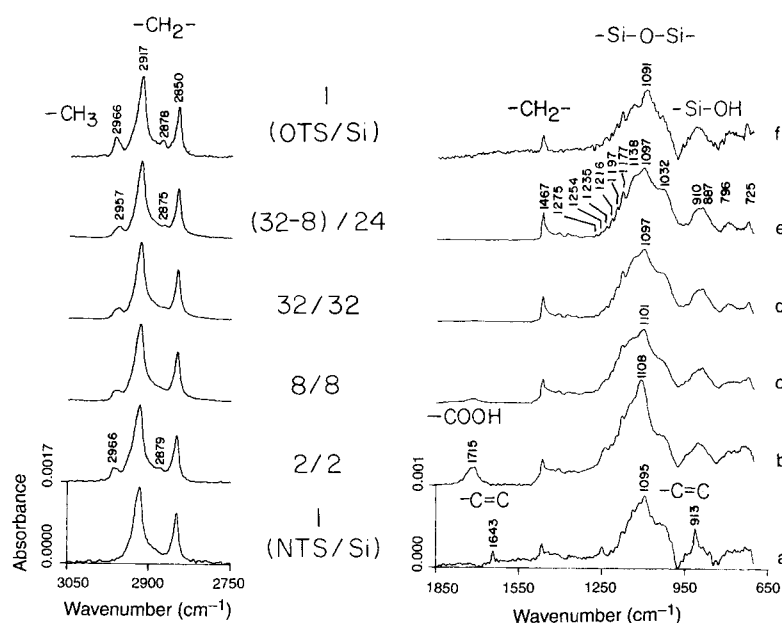


is nonadecenyltrichlorosilane—see Fig. 1.) For these bands, maximum peak intensity variations are within about ±2–3% of the average, which is the experimental reproducibility of our spectral data for such systems^{19,20}. As each of the OTS, NTS and NTS_{ox} monolayer components contains the same number (17) of H-C-H groups per molecular tail, this implies that multilayer films with the indicated nominal numbers of stacked monolayers were indeed deposited; it also means that all constituent monolayers have virtually identical density and orientation of their paraffinic tails regardless of location and total number of superimposed monolayers in the stack^{19,20}. (NTS_{ox} is the -COOH terminated silane resulting from the surface oxidation of NTS—see Fig. 1). This conclusion is corroborated by the similar behaviour observed in normal-incidence spectra of the same films (not shown), which differ from the Brewster's angle spectra (Fig. 2) in their dependence on eventual variations in the orientation of the tails. The present infrared spectra correlate quantitatively with those of films of hydrogen-bonded silanes that have been assembled layer-by-layer^{19,20}, where complete surface coverage with a packing density of 20–21 Å² per molecule was demonstrated. As before^{19,20}, the solid-like organization of the paraffinic tails is evident from both the peak positions and band widths of the -CH₂- stretch bands²¹, and the progression of weak wag and twist bands²² clearly identified between 1,275 and 1,177 cm⁻¹, which are indicative of a large proportion of hydrocarbon chains assuming an essentially all-*trans* zigzag conformation, in a dense molecular arrangement^{20–23}. The considerably reduced intensities and shifted peak positions of the -CH₃ stretch bands of OTS (2,957 and 2,875 cm⁻¹; Fig. 2 trace e), compared to those of the single OTS monolayer (Fig. 2 trace f) and halved top OTS monolayer (2,966 and 2,878 cm⁻¹; Fig. 2 trace b), are typical of overlayer-covered

methyls²³, which thus confirms the inner-film location of the OTS added to the initial bilayer (see Fig. 1).

Chemical transformations accompanying the film formation are indicated by the characteristic functional-group bands visible between 1,850 and 650 cm⁻¹ (Fig. 2). Particularly significant with regard to the proposed film growth mechanism are the -Si-O-Si- and -Si-OH bands (around 1,100 and 900 cm⁻¹, respectively), characteristic of partially condensed oligomeric polysiloxanols^{20,24}, that appear in all displayed spectral curves. Although the quantitative fit of these bands is less perfect than that of the H-C-H stretch bands, their presence in the OTS layers added to the initial bilayer implies that the -SiCl₃ functions of the trichlorosilane molecules supplied from solution undergo hydrolysis, followed by presumably lateral partial polymerization²⁰ on incorporation into the growing multilayer film (Fig. 1, top right). As no siloxane or silanol bands are detectable in the infrared spectra of presently employed OTS/BCH solutions, the water required for hydrolysis must have originated in the acetone treatment. Acetone thus functions as an appropriate water carrier, effecting the separation of the layers and the insertion of water (undried acetone contains significant amounts of water) into the interlayer polar regions of the film. The confinement of the chlorosilane hydrolysis to these polar interfaces, followed by rapid partial lateral condensation of the resulting silanols²⁰—under the chemical surface pressure set up in such circumstances at the solution-solid interface^{25,26} and the steric constraints imposed by the hydrophilic-hydrophobic segregation of the polar head groups from the non-polar paraffinic tails—are viewed as the main enthalpic/entropic driving forces for the acetone-induced formation of intercalated silane bilayers. In this manner, the acetone-mediated water supply to the preformed

FIG. 2 Examples of FTIR Brewster's-angle spectra²⁰ recorded during the assembly of a 32-layer film on each (polished) side of a silicon wafer ($4 \times 2 \times 0.038$ cm, (111) orientation, undoped), following the process described in Fig. 1. For clarity, the displayed spectral curves were shifted vertically with respect to one another (with baseline corrections for slope only), and all absorbance scales normalized to that of a single monolayer (each scale is divided by the corresponding nominal number of stacked monolayers). Trace a, the precursor NTS monolayer directly assembled on the bare Si surface (Fig. 1, inset); trace b, the initial OTS/NTS_{ox} bilayer template; trace c, eight-layer film (four bilayers) obtained after two acetone-OTS treatment cycles ($2^2 - 1 = 3$ added bilayers); trace d, the 32-layer film (16 bilayers) obtained after four acetone-OTS treatment cycles ($2^4 - 1 = 15$ added bilayers); trace e, difference spectrum approximating the spectral contribution of the inner 24 OTS monolayers added to the eight-layer film to produce the 32-layer film; trace f, a single OTS monolayer directly assembled on the bare surface of another, identically prepared, Si wafer. The spectra were taken in transmission with a p-polarized beam (electric field parallel to the plane of incidence) at the silicon Brewster's angle of incidence ($\theta_B = 73.7^\circ$), using a Nicolet 730 FTIR system equipped with a DTGS detector and a computer controlled 'shuttle' accessory which translates the sample in and out of the infrared beam at preselected time intervals, thus permitting background-corrected quasi-double-beam spectra to be recorded with a single-beam instrument. All curves are corrected for the spectral contribution of the bare Si substrate (by subtracting the spectral features recorded for the same Si wafer immediately after its cleaning and before the deposition of the first NTS monolayer) and were acquired by averaging a total of 640 scans at a resolution of



4 cm⁻¹. The Brewster's-angle configuration minimizes the interference fringes caused by the reflection of the infrared beam at the front and back Si-air interfaces of the polished-both-sides wafer, also allowing detection of vibrations perpendicular to the layer plane, which are difficult to observe in transmission at normal incidence²⁰.

bilayers triggers the addition of each new set of bilayers, their self-assembly being then controlled by the initial set to which the water was supplied. A stack of preformed bilayers thus functions as a set of independent template units which define the discrete spatial distribution of the water incorporated into the film, while providing a succession of distinct polar interfaces, each of which is capable of sustaining the spontaneous self-assembly of a similarly structured bilayer. Implicit in this model are the concept of lateral mobility in hydrogen-bonded silane monolayers^{19,20,25} and the consequent analogy to spreading Langmuir films^{25,26}, each water-containing bilayer being comparable to a 'molecular' Langmuir trough.

Acetone, water and the HCl resulting from the hydrolysis of the chlorosilane groups are not permanently retained in the film in measurable quantities, as neither the infrared nor additional X-ray photoelectron spectroscopy (XPS) measurements could reveal the presence of these species during film growth. The absence of chlorine (as shown by XPS) further confirms the complete hydrolysis of the OTS incorporated in the growing film.

Scanning force microscope (SFM) topographic images taken with a Nanoscope III instrument (operated in air in the constant-force mode) from several different film samples on silicon, at several surface spots on each of them, indicate remarkable coverage homogeneity and resistance to mechanical wear of the replicating films, independent of the total number of stacked monolayers in the film. As in single OTS monolayers, no defects, such as holes or steps, are distinguishable in any of these images and no surface defects could be induced by the tip. The outer surface corrugation of the replicating multilayers was also found to be comparable to that of the single OTS monolayers, in general following the contour of the underlying Si substrate. All this indicates that the deposited material corresponding to a given number of nominal monolayers must be evenly distributed on the surface.

X-ray reflectivity studies performed on four- and eight-layer (Fig. 3) 'self-replicating' films prepared by the present method provide a direct measure of their internal structure. The appearance of Kiessig fringes and quasi-Bragg peaks, including the $\sim 0.36 \text{ \AA}^{-1}$ peak that arises from the internal bilayer ordering

(Fig. 3), demonstrates the existence of well-defined layers whose thickness agrees with the self-replication model. Together with the SFM and FTIR evidence, and the observation of contact angles characteristic of homogeneous -CH₃ outer surfaces,^{25,26} these results indicate that the growth process indeed leads to a regular array of molecular layers in which the long molecular axes point normal to the surface.

Strong support for the proposed reactive intercalation mechanism was obtained from a series of control experiments conducted in parallel with both intercalable and non-intercalable films. Following identical acetone treatments there was no deposition of additional OTS material on a preformed OTS/Si monolayer or a OTS/NTS_{ox} bilayer with interlayer spacing fixed by partial covalent bonding, whereas an ordered OTS trilayer was deposited on top of a preformed NTS_{ox}/Si monolayer. In the latter case, the initial fast formation of a water-containing OTS/NTS_{ox} bilayer would permit the further rapid intercalation of an additional OTS bilayer provided that a suitable surplus of water adsorbed on the preformed NTS_{ox} monolayer outlives the fast deposition of OTS on top of it. Attempts to trigger replicative film growth using water itself rather than acetone failed, as water from the bulk liquid is not incorporated in such films²⁰.

Our results described experiments were reproduced with about ten different film samples on Si and quartz having lateral dimensions between 1×1 cm to 2×5 cm. These observations establish that, unlike simple crystal growth, the film growth described here is discontinuous and involves two consecutive chemical reactions (hydrolysis and condensation) taking place within the boundary of the film itself as a precondition for the spontaneous organization of the reacted material into a structure akin to that of the initial film. The entire process is made possible by the specific organization and properties of a pre-existing structure which acts both as a host micro-reactor and template for its own replication^{27,28}. This replicating system is 'non-informational', in the sense of a polymer made up from different monomers¹¹; however, it carries structural information enabling the replication of an entire solid-immobilized supramolecular assembly, rather than that of single molecules^{11,12} or floppy aggregates in solution^{12,28}. The necessary separation of the original and its replica^{11,12,27} (here indistinguishable

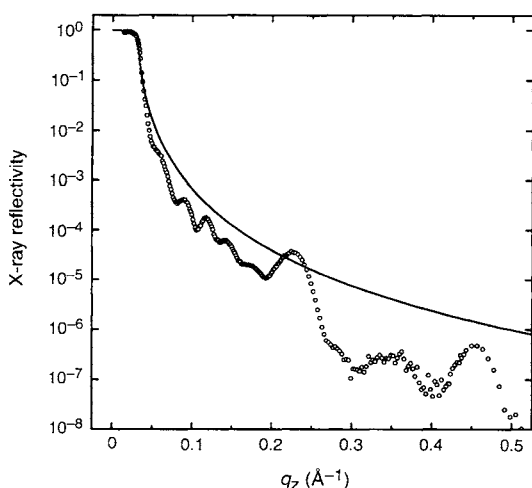


FIG. 3 X-ray reflectivity (measured at beamline X22B at the National Synchrotron Light Source) versus q_z for a self-replicating eight-layer film on Si prepared as the films of Fig. 2 (circles). (q_z is the wavevector transfer along the surface normal.) The reflectivity decreases faster at large q_z than that of a perfectly flat Si interface (Fresnel theory, solid line), indicating a substantial substrate roughness of the order of 5 Å. Oscillations in the reflectivity are due to the interference between rays reflected from the top and bottom of the film. The existence of multiple oscillations shows the presence of well ordered layers. The total film thickness derived from the 0.028 Å^{-1} spacing of these Kiessig fringes is $224 \pm 8 \text{ Å}$. The quasi-Bragg peaks at ~ 0.23 and $\sim 0.45 \text{ Å}^{-1}$ indicate a layer spacing of 28 Å , close to the expected thickness of an untilted OTS monolayer²⁰. Thus, the ratio of the total thickness to the layer spacing (8.0 ± 0.3) confirms the existence of eight silane layers, in accordance with the self-replicating growth model. The broad, weaker peak at $\sim 0.36 \text{ Å}^{-1}$ originates from the presence of the OTS bilayers (Fig. 1) and is absent in the reflectivity of NTS films with same number of layers grown layer-by-layer (not shown)²⁰. The reflectivity results for a four-layer film show that the thickness is half that of the eight-layer film; but a detailed interpretation is slightly complicated by the fact that, for technical reasons, the quasi-Bragg peak is far less pronounced, and so the data are not shown here.

from the copy²⁹) is achieved with conservation of the overall structural integrity of the system, which leads to the growth of a three-dimensional structure (multilayer), starting from an initial two-dimensional nucleus (monolayer) itself generated via controlled self-assembly at the interface. Thus, unlike the related self-multiplication previously observed in some layer silicates²⁷, the present process does not rely on the availability of a template of natural origin. Our system contains the instructions required for its own replication while still offering the advantage that the replication is discontinuous and subject to precise control through an external chemical trigger operation. ■

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Effect of slab temperature on deep-earthquake aftershock productivity and magnitude–frequency relations

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DEEP earthquakes generally show fewer aftershocks and a larger variability in magnitude–frequency relations than shallow earthquakes^{1–5}. These characteristics and the factors that control them may place constraints on the mechanism of deep earthquakes, which is still uncertain^{6–8}. Here we show that systematic variations in aftershock productivity and magnitude–frequency relations are related to the temperature of the downgoing slab, as inferred from the vertical velocity and age of the subducting lithosphere. Deep earthquakes with substantial aftershock sequences are found only in colder slabs, and earthquakes in warmer slabs consistently show few aftershocks. Magnitude–frequency relations for deep earthquakes also show systematic variations as a function of slab thermal structure, with warmer slabs showing fewer small earthquakes (lower ‘*b*-values’). The statistical properties of deep earthquakes thus appear to be temperature sensitive to an extent not observed for shallow earthquakes, and not adequately explained by simple geometrical limitations on fault width.

The aftershock productivity of deep earthquakes varies from a nearly complete absence of aftershocks to aftershock rates approaching those of typical shallow earthquakes. As extreme examples, the 1970 Colombia deep earthquake (moment magnitude $M_w = 8.0$) produced no reported aftershocks, whereas 144 aftershocks have recently been recorded for the much smaller 9 March 1994 Tonga event ($M_w = 7.6$)^{9,10}.

To address the systematics of deep-earthquake aftershock production, we have compiled two datasets. One dataset consists of all 1958–96 deep earthquakes with $M_w \geq 7.0$ (Table 1a). For this dataset we compiled all reported aftershocks with body wave

magnitude (m_b) ≥ 4.5 with the magnitude threshold imposed to reduce the effect of uneven detection levels. Events are counted as aftershocks if they occurred within 100 km of the mainshock hypocentre within a period of 3 months following the mainshock. For most cases, this simple criterion readily distinguishes aftershocks from the background seismicity, particularly because most deep seismic zones show relatively low seismicity levels. For a few active deep zones, seismicity rates are high enough that this criterion might include several randomly occurring background events. In these cases, the time window is limited to the period in which the seismicity rate exceeds the background seismicity rate.

We also compiled a second dataset to take advantage of the superior instrumentation available for recent deep earthquakes. This dataset consists of 1990–96 deep earthquakes with $M_w \geq 6.0$, for which waveform data is available from at least one station within 12° distance (Table 1b). For the 1994 Tonga and Bolivia events we used results from temporary networks operating in these regions^{9,11}. For other events, we obtained data from all nearby broadband stations and identified any aftershocks within a 2-day period following the mainshock. Any event showing P–S

times similar to the mainshock was considered an aftershock. These aftershocks were added to any identified from the monthly Preliminary Determination of Epicenters (PDE) bulletin using the criteria noted above. If there were any appreciable aftershocks, the broadband data were searched for an additional time period. This procedure will identify any significant aftershock sequences, as the occurrence rate of aftershocks decays rapidly following the mainshock^{9,12,13}. Because the deep earthquakes in this study vary in seismic moment by more than three orders of magnitude, the aftershock production must be corrected for differences in mainshock size. We normalize the number of aftershocks assuming that the number of aftershocks scale with M_w (ref. 5). The normalization simply permits results from a wider range of mainshock magnitudes to be compared simultaneously.

The results, when tabulated, suggest that aftershock production varies systematically between different subduction zones. Although there is substantial variability, the Tonga and Marianas subduction zones show more aftershocks on average than subduction zones in South America for deep earthquakes of equivalent size. Deep earthquakes in the Kurile, Japan, and Izu–Bonin regions also show few aftershocks.

Although the results shown in Fig. 1a have been normalized to permit comparison of a wide range of mainshock magnitudes, the differences in aftershock production are clear in the raw aftershock numbers. For example, the 1996 Indonesia event (M_w 7.8) in a relatively cold slab shows 14 aftershocks ($m_b \geq 4.5$), whereas the larger 1970 Colombia event (M_w 8.0) in a warm slab shows no aftershocks. The 1996 Indonesia event can also be compared to the 1973 Japan event (M_w 7.7) and the August 1963 Peru event (M_w 7.7), both of which occur in warmer slabs and also show no aftershocks. The 1994 Tonga event (M_w 7.6) in the coldest slab shows 19 aftershocks ($m_b \geq 4.5$), whereas the November 1963 Peru event with the same moment magnitude shows two aftershocks.

It may be thought that these trends reflect the detection levels in the various subduction zones. The converse is true, as detection levels in the Tonga and Marianas subduction zones are worse than in most other subduction zones. Of the subduction zones studied, the Tonga subduction zone shows one of the highest detection threshold levels, as indicated by changes in the slope of the magnitude–frequency curve. For example, only 60% of the $m_b \geq 4.5$ aftershocks from the 9 March 1994 Tonga event were detected by the PDE, as determined by comparison with the local broadband data.

Aftershock productivity shows a strong relationship to the thermal

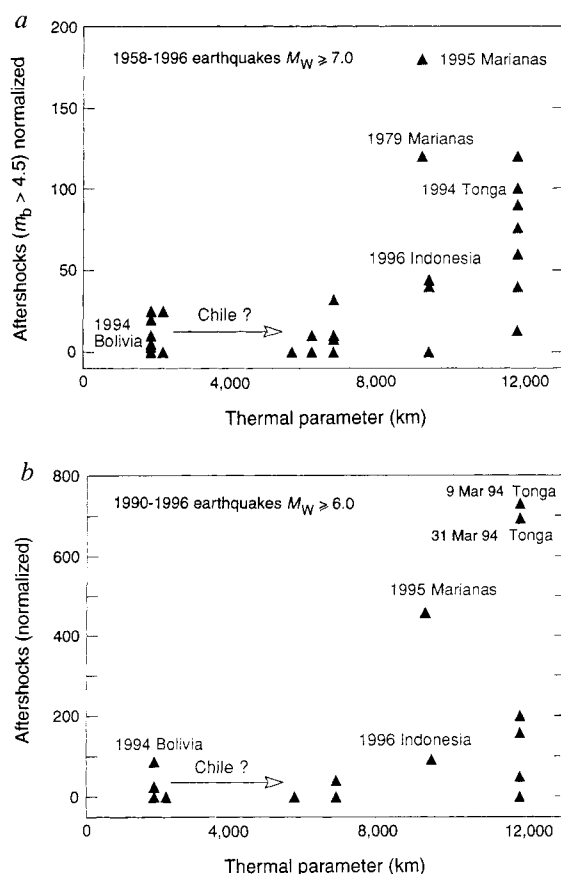


FIG. 1 a, Number of normalized aftershocks ($m_b \geq 4.5$) for large 1958–96 deep earthquakes (Table 1a) as a function of subduction-zone thermal parameter (product of vertical descent rate and the age of the subducting lithosphere). Larger thermal parameters correspond to cooler slabs. Uncertainty for the thermal parameter for Chile is shown by the arrow; thermal parameters as high as 6,500 km are possible if the deep seismic zone occurs within Mesozoic age lithosphere¹⁶. The number of aftershocks are normalized to correct for different mainshock sizes assuming that the logarithm of the number of aftershocks should be proportional to M_w , as is observed for shallow earthquakes. b, Total number of normalized aftershocks recorded for 1990–96 deep earthquakes (Table 1b) as a function of subduction-zone thermal parameter. Data is shown only for earthquakes for which the number of aftershocks could be checked by at least one broadband seismic station within 12° distance. Deep earthquakes in cold slabs show a much higher aftershock rate for both datasets.

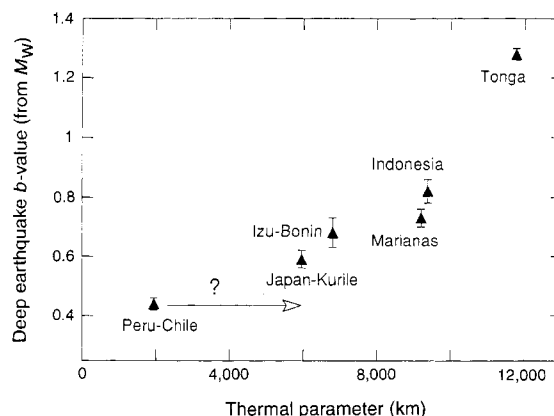


FIG. 2 The b-value determined from the magnitude–frequency relation for various deep seismic zones as a function of slab thermal parameter. The uncertainty for the thermal parameter of the Peru–Chile deep seismic zone is shown by the arrow. Cold deep seismic zones show a much higher b-value than warmer slabs, showing that larger earthquakes predominate in warmer slabs and demonstrating that the statistical behaviour of deep earthquakes is temperature dependent.

characteristics of the slab. Figure 1 plots the normalized number of aftershocks as a function of thermal parameter for both datasets. The thermal parameter, the product of the slab vertical-descent rate and the age of the subducting lithosphere, is a simple way of estimating the overall temperature structure of the deep slab. The use of the thermal parameter to compare temperatures at depth in the slab is justified theoretically if heating of the subducted lithosphere occurs by conduction and if the lithospheric temperature structure is given by a halfspace cooling model before subduction¹⁴. Larger thermal parameters correspond to cooler slab temperatures at depth. Table 2 lists the values used to calculate the thermal parameters for each subduction zone.

For several regions, some complicating factors must be taken into account in calculating the thermal parameter. The Tonga, Marianas and Izu–Bonin regions have active back-arc spreading systems, and so estimates of the back-arc spreading rate from marine surveys are added to the convergence rate of the major plates. Recent GPS surveys of the Tonga region¹⁵ suggest an exceedingly fast convergence rate ($\sim 21 \text{ cm yr}^{-1}$). This rate is probably not indicative of the descent rate of the deep slab, so we use the absolute velocity of the Pacific plate to estimate the vertical descent rate of the steeply dipping deep part of the slab. For the South American subduction zones, uncertainty in the age of the lithosphere at depth complicates the calculation. It has been suggested that the material at depth may be as much as 100 Myr older than the currently subducting plate¹⁶. Plate reconstructions suggest that this is not the case for the deep seismic zone in the Peru region¹⁷, but it is less clear for Chile. Use of the older lithospheric ages would raise the thermal parameter from about 2,200 to 6,500 km; the uncertainty in this parameter for Chile is shown by an arrow on the figures.

The maximum aftershock productivity is correlated with the slab thermal parameter for both datasets (Fig. 1). Deep earthquakes showing substantial aftershock productivity are limited to colder slabs, such as Tonga and the Marianas. Warmer slabs, such as Chile and Peru, consistently show few aftershocks. This suggests that the temperature of the slab plays a very important role in controlling

TABLE 1 Aftershocks of deep earthquakes

Date	Subduction zone	Depth (km)*	M_w †	Number‡	Normalized aftershocks§
<i>a</i> Aftershocks ($m_b \geq 4.5$) for 1958–96 deep earthquakes ($M_w \geq 7.0$)					
9 June 94	Bolivia	636	8.3	3	3
31 Jul 70	Colombia	651	8.0	0	0
17 Jun 96	Indonesia	565	7.8	14	44
15 Aug 63	Peru	543	7.7	0	0
29 Sep 73	Japan	575	7.7	0	0
26 Jul 58	Peru–Chile	650	7.6	1	5
9 Nov 63	Peru	600	7.6	2	10
9 Mar 94	Tonga	565	7.6	19	95
22 Jun 82	Indonesia	450	7.4	0	0
6 Mar 84	Izu–Bonin	457	7.4	1	8
7 Oct 68	Izu–Bonin	457	7.3	1	10
30 Aug 70	Kurile	645	7.3	1	10
21 Jul 94	Japan	473	7.3	0	0
31 Aug 61	Peru	626	7.2	2	25
9 Oct 67	Tonga	654	7.2	6	76
10 Feb 69	Tonga	673	7.2	1	13
11 Feb 69	Indonesia	450	7.2	0	0
30 Mar 72	Tonga	532	7.2	6	76
12 May 90	Kurile	611	7.2	0	0
23 June 91	Chile	581	7.2	2	25
8 Dec 62	Chile	620	7.1	0	0
29 Nov 74	Izu–Bonin	419	7.1	2	32
29 Jun 75	Japan	560	7.1	0	0
7 Mar 78	Izu–Bonin	442	7.1	0	0
26 May 86	Tonga	553	7.1	6	95
25 Aug 63	Tonga	557	7.0	2	40
15 Dec 63	Indonesia	650	7.0	0	0
23 Mar 74	Tonga	535	7.0	6	120
17 Oct 79	Marianas	601	7.0	6	120
16 June 86	Tonga	565	7.0	3	60
5 May 89	Peru	604	7.0	1	20
24 May 90	Indonesia	588	7.0	2	40
23 Aug 95	Marianas	595	7.0	9	180
<i>b</i> Aftershocks for 1990–96 deep earthquakes ($M_w \geq 6.0$)					
9 June 94	Bolivia	636	8.3	87	87
17 June 96	Indonesia	565	7.8	33	104
9 Mar 94	Tonga	565	7.6	144	722
21 Jul 94	Japan	473	7.3	0	0
23 Aug 95	Marianas	595	7.0	23	459
17 Oct 90	Peru	624	6.9	1	25
10 Jun 94	Peru	589	6.9	0	0
29 Apr 94	Chile	573	6.9	0	0
10 May 94	Chile	605	6.9	0	0
3 May 91	Izu–Bonin	459	6.7	1	40
20 Jun 92	Izu–Bonin	512	6.6	0	0
27 Oct 94	Tonga	549	6.6	1	50
19 Jan 93	Japan	455	6.5	0	0
31 Mar 94	Tonga	591	6.5	11	694
30 Oct 92	Isu–Bonin	406	6.4	0	0
19 Aug 94	Chile	565	6.4	0	0
17 Jan 95	Tonga	637	6.3	2	200
4 Nov 94	Peru	618	6.1	0	0
29 Oct 95	Tonga	611	6.1	1	158
19 Jan 94	Tonga	537	6.0	0	0

* Deep earthquakes are defined as deeper than 400 km for this study.

† Moment magnitude calculated from ref. 32 for 1958–61 earthquakes, from ref. 33 for 1962–76 earthquakes and from Harvard CMT solutions³⁴ for 1977–96 events.

‡ For *a* the number of aftershocks $m_b \geq 4.5$ from the Preliminary Determination of Epicenters (PDE) and the International Seismological Centre (ISC) are tabulated; for *b* the total number of aftershocks determined from all sources are tabulated. With the exception of 17 Jun 96, only events for which broadband waveform data was available from stations within 12° distance are included in *b*. The total number of aftershocks for 9 Mar 1994 are from ref. 10, and for 9 Jun 94 the number of aftershocks are taken from ref. 11.

§ Aftershocks are normalized assuming that the log of the number of aftershocks scales linearly with M_w . This relationship is consistent with aftershock sequences of shallow earthquakes in both California and Japan²⁶. Aftershocks are normalized to the number expected for an M_w 8.3 earthquake using $\log N_p = 8.3 - M_w + \log N_o$, where N_p and N_o are the normalized and observed number of aftershocks respectively⁵.

|| Aftershock statistics for the recent 17 June 96 event are determined from the Reviewed Event Bulletin (REB) and the weekly PDE, as the monthly PDE data is not yet available. A magnitude correction is made to events located only by the REB. As no data are available from within 12° , the number of aftershocks for this event may be underestimated relative to the others in *b*.

TABLE 2 Subduction zone parameters

Subduction zone	Age (Myr)*	Velocity (mm yr ⁻¹)†	Backarc velocity‡	Angle (deg)§	Vertical velocity	Thermal parameter	b-value
Kurile	100	82	0	49	62	6,200	0.59 (± 0.03)
Japan	130	87	0	30	43	5,600	—
Izu-Bonin	145	55	5	51	47	6,800	0.68 (± 0.05)
Marianas	155	44	22	64	59	9,200	0.73 (± 0.03)
Indonesia	140	79	0	58	67	9,400	0.82 (± 0.04)
Tonga	110	84	130	53	107¶	11,800	1.28 (± 0.02)
Peru	41	78	0	35	44	1,800	0.44 (± 0.02)
Chile	45	84	0	35	48	2,200	—

* Age of the lithosphere entering the trench, from compilations of magnetic anomalies³⁵ and the magnetic timescale³⁶.

† Relative velocity at the trench³⁷.

‡ Backarc spreading rates taken from ref. 38 for the Marianas, ref. 39 for Izu-Bonin, and ref. 15 for Tonga.

§ Average slab dip, obtained by calculating the angle subtended by a line connecting the trench axis with the deep seismicity.

|| Slope of the magnitude–frequency curve determined by least squares from 1977–95 M_w values (taken from the Harvard CMT catalogue³⁴ over an M_w range of 5.3–6.8. Adjacent subduction zones with similar thermal parameters were combined for the Peru–Chile and Japan–Kurile regions to obtain enough samples for the b -value determination. The uncertainty is the least-squares standard deviation.

¶ Owing to slab rollback and the complex recent history of the Tonga slab, the vertical velocity is set to the absolute motion of the Pacific plate⁴⁰, to approximate the velocity of the descending slab relative to the mantle. Use of the complete convergence rate would result in a much larger thermal parameter.

the mode of seismic energy release in aftershock sequences, and that aftershock sequences may be particularly suppressed in warm sections of the slab. This observation is consistent with the characteristics of the large 1994 Tonga deep earthquake, which shows few aftershocks in the outermost, warmer region of the slab⁸.

Previous studies have noted that deep earthquakes in different subduction zones show different slopes of the magnitude–frequency relation (b -value)^{1,3–5}. We determined the b -values for the deep seismic zones studied here from a least-squares fit to 1977–95 M_w data, with M_w values determined from the Harvard centroid moment tensor (CMT) dataset. The Chile–Peru and Japan–Kurile regions were combined to obtain enough moment data to determine a b -value, as they are adjacent and have similar thermal parameters. The resulting b -values are also correlated with the thermal parameter (Fig. 2), suggesting that slab thermal structure also controls the magnitude–frequency relation for deep earthquakes. Not surprisingly, the subduction zones showing the largest b -values also tend to show greater aftershock activity⁵. A similar correlation is obtained using b -values determined from more numerous m_b data.

A previous study⁴ has proposed that the difference in b -values between Tonga and other slabs is due to the effect of the finite width of slab material available for faulting, as has been observed for very large shallow earthquakes^{18,19}. The present study shows that slab thermal structure significantly affects aftershock occurrence for equivalently sized faults. This effect cannot result from

simple physical limits on the width of faults, which should not cause different aftershock levels for similar sized faults in different subduction zones. Rather, this study suggests a more fundamental effect of temperature on the statistics of deep earthquake faulting, including both magnitude–frequency relations and aftershock productivity.

Although some studies suggest small regions of anomalous magnitude–frequency relations at shallow or intermediate depth^{20–22}, shallow earthquakes generally show remarkably uniform b -values^{3,23}. Similarly, although some differences in aftershock and foreshock production have been reported for shallow events^{24,25}, other studies find shallow aftershocks consistently proportional to fault area²⁶, and no systematic aftershock production anomalies have been definitively established¹³. This study demonstrates that aftershock production and magnitude–frequency relations for deep earthquakes are temperature dependent in a fundamentally different way from shallow earthquakes.

The observation that deep earthquake statistical properties are temperature dependent provides important constraints on deep-earthquake generating processes. Several proposed mechanisms for deep earthquakes are expected to be highly temperature sensitive, beyond the obvious temperature effect of limiting slab width. One possible candidate is plastic shear instabilities^{27,28}, which are likely to be highly temperature dependent. These instabilities may be triggered in the slab core by transformational faulting^{29–31}. □

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A *Pikaia*-like chordate from the Lower Cambrian of China

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THE earliest evolution of the chordates and their relationships with other deuterostomes remains controversial¹⁻³. Rejuvenation of interest in the areas of molecular phylogeny⁴ and developmental genetics⁵⁻⁷ has not been matched by new insights from the fossil record, which for the Lower and Middle Cambrian remains exceptionally sparse. The supposed chordate *Emmonsaspis*⁸ has been shown to be a frond-like fossil⁹, and phosphatic sclerites of hadimopanellids, once compared with vertebrate dermal armour^{10,11}, are now known to be derived from the cuticle of protostome palaeoscolecoidan worms^{12,13}. Other fragmentary material of supposed scales is even more dubious¹⁴. The best-known candidate in this thin roster remains the Burgess Shale chordate *Pikaia*¹⁵. Here we report a single specimen of a Lower Cambrian chordate, *Cathaymyrus diadexus*, new genus and species, that is similar to *Pikaia* but predates it by about 10 million years (Myr). Important features of this new specimen are structures interpreted as pharyngeal gill slits. The evolution of chordates was an integral part of the first stages of the Cambrian 'explosion', and steps to craniates with neural crest were probably achieved by the Middle Cambrian.

A single example (part and counterpart) of an early chordate from the Chengjiang Lagerstätte of Yunnan, China, was collected in 1995. This deposit is already celebrated for its superbly preserved soft-bodied fauna^{16,17}, which is similar to the Middle Cambrian Burgess Shale¹⁸. This animal is rare: so far only one specimen has been recognized in collections that now exceed 10,000 specimens. The specimen (Fig. 1a) is about 22 mm long, with a sinuous eel-like disposition. The anterior appears to show a modest expansion and contains a concave ovoid area, still partially filled with sediment. This structure presumably represents the pharynx. Closely spaced (about 16 per mm) transverse striations on the pharynx (Fig. 1b) are interpreted as gill slits (Fig. 2). The remainder of the body is segmented, and although faintly preserved, the arrangement of the transverse structures is consistent with that of myotomes, separated by the myocommata. Although their sigmoidal shape is clear, the quality of preservation and the possibility of superposition of either side makes a description of the precise configuration difficult. Evidence for an abrupt geniculation in some myotomes (Figures 1a and 2) may, however, be consistent with their having a more complex shape than the simple vee-shapes that occur in amphioxus. A rather prominent but narrow ridge runs longitudinally. This may be a product of compaction, or conceivably it represents an internal structure such as the notochord, which in amphioxus is known to be relatively resistant to decay¹⁹. If it is the notochord then it appears to terminate short of the head region. Parallel to this prominent ridge is a much fainter and more discontinuous structure. This could be an internal organ such as the gut. The posterior terminates in a fine tip. In this specimen there is no evidence for either fins or fin rays. Alternative explanations, notably that the structures interpreted here as gill slits are in fact fin rays similar to those in the tail of hag fish, remain possibilities, but are considered less likely.

This Chengjiang fossil has a striking resemblance to the Burgess Shale animal, *Pikaia gracilens* (Fig. 1c), generally accepted as a primitive chordate¹⁵. Similarities include overall shape, anterior pharynx, whose sediment-filled nature indicates an original spaciousness, myotomes, and possible notochord. Features equivalent to the structures interpreted as gill slits, however, have not

been clearly observed in *Pikaia*, although the preservational history of the Burgess Shale is different from the Chengjiang sediments on account of the former experiencing a significantly greater degree of compaction and heating, leading to what is effectively a metamorphic rock²⁰. Future discoveries from Chengjiang or nearby localities should resolve whether the similarities to *Pikaia* include a distinctive bilobed head with narrow tentacles and a series of short appendages extending from either side of the anterior region¹⁵. The connection, if any, of these short appendages with the presumed pharyngeal gill slits is uncertain. They are much more widely spaced than the transverse structures in the Chengjiang animal (Fig. 1b), and in *Pikaia* these appendages also

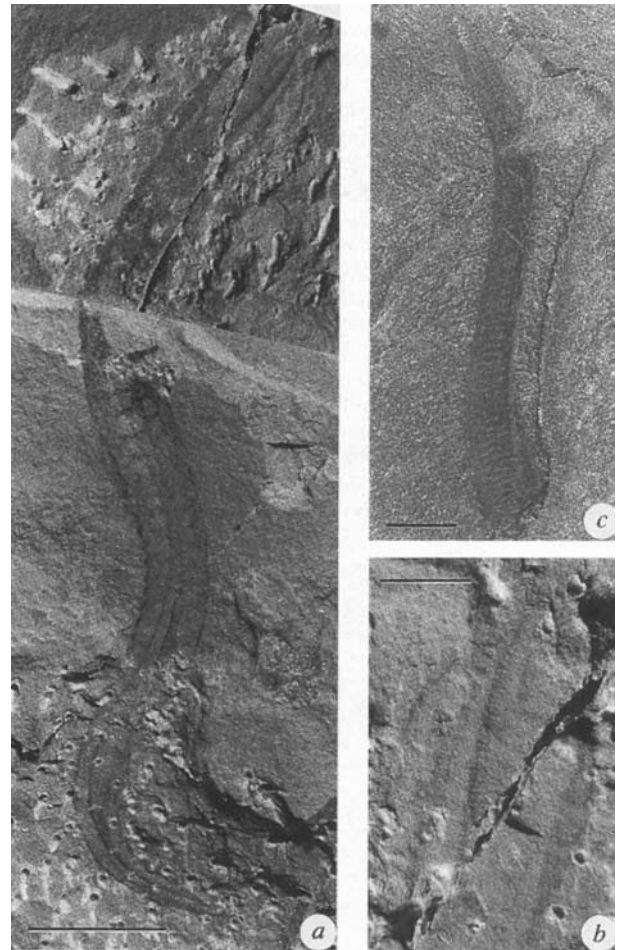


FIG. 1 The Cambrian cephalochordates *Cathaymyrus diadexus*, new species, and *Pikaia gracilens* Walcott. The specimen from the Chengjiang lies obliquely to the plane of bedding so that the anterior and posterior ends of part and counterpart, respectively, required excavation. In many specimens of *Pikaia*, including USNM 198694, parts of the body are folded beneath other regions. This is a result of the body being laterally flattened and transported in a turbulent mud-flow. a, *Cathaymyrus diadexus*, new species, NWU 95-1405, composite photograph of part and counterpart. Anterior region (compare with Fig. 2) has been printed with negative reversed and then mounted against the unreversed posterior region which remains, therefore, in its correct configuration. The reasons for this is that the oblique nature of the split resulted in the part consisting of the anterior half and the corresponding counterpart to the posterior half of the specimen. Only a limited region of the mid-section of the body was originally visible in part and counterpart, and mechanical excavation in either direction was necessary to expose the entire specimen. Scale bar, 3 mm. b, *Cathaymyrus diadexus*, new species, detail of anterior region including pharynx and gill slits. Photograph is in correct (unreversed) orientation. Scale bar, 1 mm. c, *Pikaia gracilens* Walcott, USNM 198694, notochord runs along the right-hand side. Note also the myotomes. Posterior of body is bent beneath rest of body. Scale bar, 5 mm.

extend posterior to the sediment-filled region interpreted as the pharynx. If connected, they may represent exhalant openings. Evidence for the notochord stopping well short of the anterior in *Pikaia*¹⁵ (Fig. 1c), and possibly this Chengjiang animal (Figs 1a and 2), may be significant. In amphioxus, the notochord only extends into the rostral region later in embryology²¹, even though *Brachyury* transcripts are expressed along the entire length of the notochord⁷.

A detailed consideration of the place of the Cambrian taxa *Cathaymyrus* and *Pikaia* in early chordate phylogeny awaits further discoveries of the former animal and/or a detailed assessment of the hundred-plus specimens of *Pikaia* (S.C.M. and D. Collins, manuscript in preparation). Although the living cephalochordates are known only from the two genera grouped as the amphioxus animal, on present evidence it seems legitimate to include provisionally *Cathaymyrus* and *Pikaia* in this subclass as they appear to lack tissues associated with the neural crest. The amphioxus animal would retain its status as the most primitive living chordate, but known differences with the Cambrian taxa, such as anterior extent of the notochord and disposition of gill slits, would indicate its own degree of specialization. Concerning the calcichordate hypothesis, which remains very controversial²², this discovery of a Lower Cambrian chordate emphasizes the present incongruity between interpretation of calcichordate anatomical characters (and their placement in a particular cladistic framework³) and the stratigraphic evidence that points to the emergence of the chordates substantially earlier in the Cambrian.

Recently, doubt²³ has been thrown on the thesis that the Lower Cambrian fossil *Yunnanozoon lividum*, also from Chengjiang, is a chordate^{24,25}. For example, the supposed notochord appears to have gut contents. In addition, the putative myotomes have no indication of the 'cone-in-cone' musculature seen in chordates (or its sigmoidal surface expression that is clearly expressed in *Cathaymyrus* and *Pikaia*), but transpire to be cuticularized seg-

ments with clear evidence of post-mortem wrinkling. The specimen described here also gives no support to the earlier interpretation of *Yunnanozoon* as a chordate²⁴⁻²⁶. An affinity of this taxon with the hemichordates²³ fits the present evidence best, but even this hypothesis may require some further consideration. Why, for example, are the structures interpreted as gill slits so widely spaced in comparison with the balanoglossid acorn-worms, amphioxus, and this Chengjiang chordate?

The Chengjiang biota is generally regarded as late Atdabanian, although some correlations indicate a Botomian-equivalent age²⁷. Thus, perhaps 10 Myr separate this assemblage from the time of deposition of the Burgess Shale. Burgess Shale-type faunas, however, show a pronounced conservatism¹⁸ and the evolutionary steps to the craniates with neural crest tissue were probably well underway during this geological interval. This is because of the recognition of the conodonts as vertebrates^{28,29}, and the evidence that the paraconodonts, which appeared in the Middle Cambrian, are the direct evolutionary predecessors of conodonts³⁰. It also remains to be seen what fossil evidence may become available concerning both the hemichordate/echinoderm-chordate transition and, more importantly perhaps, the deuterostome-protostome divergence.

Phylum Chordata
Class Cephalochordata
Cathaymyrus diadexus gen. et sp. nov.

Diagnosis. Angulliform cephalochordate with anterior, possibly expanded, containing large pharynx and associated closely spaced gill slits. Trunk with myomeres, tapering to fine point.

Type (and only known) species. *Cathaymyrus diadexus*.

Etymology. *Myrus* (Latin) refers to the fossil's eel-like shape, *Cathay* to its provenance in China. Greek *diadexus*, presaging good luck, in anticipation of future discoveries.

Holotype. Department of Geology, Northwest University, Xi'an: NWU 95-1405.

Stratigraphy and locality. Qiongzhusi (Chiungchussu) Formation, Yu'an-shan Member (*Eoredlichia* Zone); Lower Cambrian. Specimen collected from the Ma'an-shan section, a new productive locality of the Chengjiang Lagerstätte, situated 3 km north of the classic Maotianshan section. □

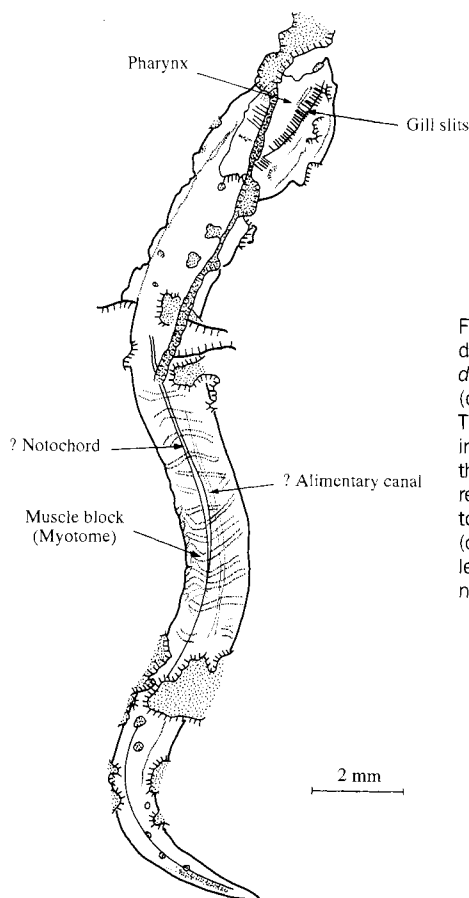


FIG. 2 Camera lucida drawing of *Cathaymyrus diadexus*, new species (compare with Fig. 1a). This interpretative drawing is a composite, with the anterior section (part) reversed and connected to the posterior section (counterpart). See Fig. 1 legend for a full explanation.

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A new brain region for coordinating speech articulation

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HUMAN speech requires complex planning and coordination of mouth and tongue movements. Certain types of brain injury can lead to a condition known as apraxia of speech, in which patients are impaired in their ability to coordinate speech movements but their ability to perceive speech sounds, including their own errors, is unaffected¹⁻³. The brain regions involved in coordinating speech, however, remain largely unknown. In this study, brain lesions of 25 stroke patients with a disorder in the motor planning of articulatory movements were compared with lesions of 19 patients without such deficits. A robust double dissociation was found between these two groups. All patients with articulatory planning deficits had lesions that included a discrete region of the left precentral gyrus of the insula, a cortical area beneath the frontal and temporal lobes. This area was completely spared in all patients without these articulation deficits. Thus this area seems to be specialized for the motor planning of speech.

Stroke, head trauma, tumour or other neurological diseases can disrupt the articulation process. One such disruption, apraxia of speech, is a disorder in programming the speech musculature to produce the correct sounds of words in the proper sequence with the appropriate timing. Patients with this deficit make inconsistent articulatory errors approximating the target word, show articulatory groping, and often have an associated disruption in prosody and rate of speech (Box 1). Some consider apraxia of speech to be a disorder in temporal coordination in which 'the timing or integration of movements of two independent articulators' is affected (ref. 1, page 162). The disorder is not considered a

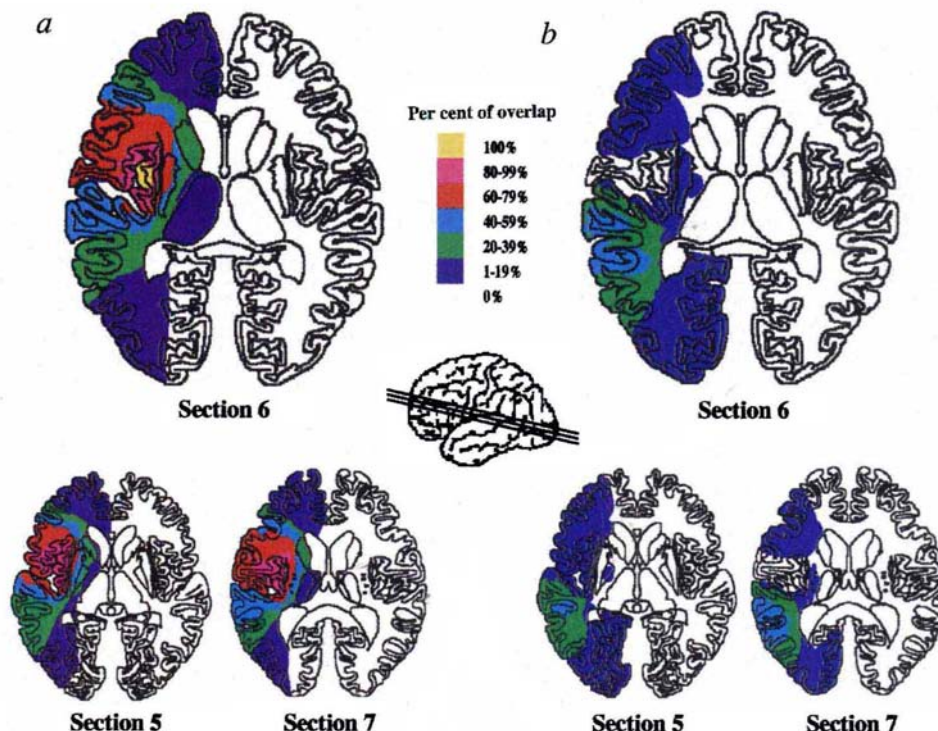
perceptual problem, as patients with apraxia of speech exhibit articulatory deficits in the absence of any difficulty in perceiving or recognizing speech sounds, including their own articulatory errors^{2,3}.

Computer reconstructions and overlapping of focal brain lesions derived from computerized axial tomography (CT) and magnetic resonance imaging (MRI) can help identify common areas of infarction in stroke patients with this and other disorders. In this study, lesions derived from CT and MRI scans of 25 patients with apraxia of speech were reconstructed onto templates and entered into a minicomputer using software and techniques developed at the VA Medical Center in Martinez, California⁴. Lesion overlays were produced by computer-superimposing lesions represented on each horizontal slice for all patients demonstrating the same articulatory deficit. In this manner, the extent to which all patients with apraxia of speech share a common lesion site could be determined.

One common area of infarction was found in all 25 cases. Figure 1a (top) depicts the lesion overlay for section 6 of the reconstruction program, containing the only region in which every patient had sustained injury. This area of 100% lesion overlap is shown in yellow and signifies the involvement of that region in each patient. It does not include any traditionally known language area, but lies within the insula, an island of cortex within the cerebral hemispheres. This common area of injury does not involve the entire insula but is confined to one discrete site and suggests the specialization of this area for the planning of articulatory movements. Although the association of a lesion site with a behavioural deficit is not conclusive proof that the site actually carries out that function, the strength of this particular association is difficult to ignore. Neighbouring sections 5 and 7 (Fig. 1a, bottom) also depict portions of the insula with 80-99% lesion overlap, but do not show a discrete area of infarction common to all patients as in section 6.

Given the significance of this finding, a means of corroborating these results was sought. A second group of 19 patients was selected who met similar selection criteria to the previous group. However, in this second group of patients similarly evaluated, none suffered from apraxia of speech. Lesion reconstructions in this group of patients were also overlapped to determine

FIG. 1 Comparison of computerized lesion overlapping in patients with and without apraxia of speech. *a*, Overlapping the lesions of 25 patients with apraxia of speech yields a common area of infarction (bright yellow) on section 6 of the reconstruction programme (top of the figure). This region represents an area of 100% overlap, that is, all 25 patients with this articulatory planning disorder have lesions that include this one area of the insula. In the bottom row are shown neighbouring sections either below (section 5) or above (section 7) the critical slice. *b*, Overlapping the lesions of 19 patients without apraxia of speech shows infarctions over much of the left hemisphere as in the previous group. However, a comparison of section 6 in the two groups reveals that not one of these 19 patients without apraxia of speech has a lesion in the same region of the insula as those who do exhibit the disorder. (The additional sparing of neighbouring motor face cortex is due to patterns of vascularization and stroke in this region.)



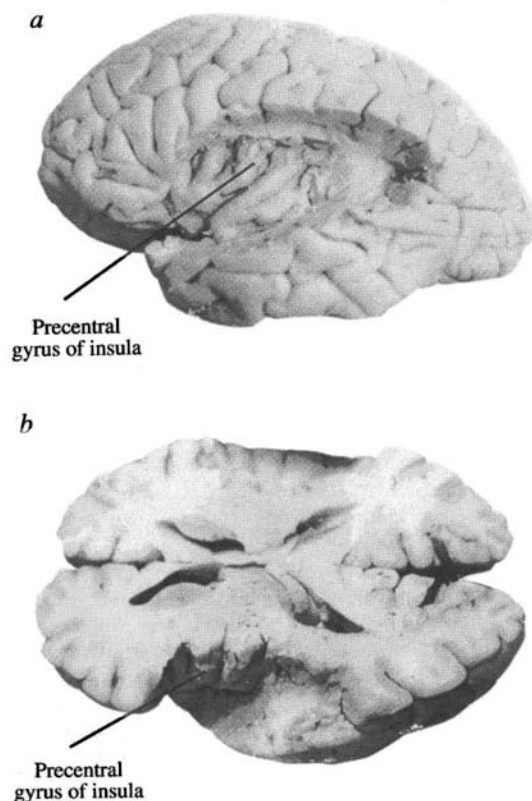


FIG. 2 Dissecting the brain at the same angle as the reconstruction templates revealed the location of the critical area for articulatory planning on the precentral gyrus of the insula. *a*, The insula lies exposed after cutting away the inferior frontal and parietal lobes and the anterior superior temporal lobe which collectively hide the insula. With this area now visible, the central insular (longitudinal) sulcus, separating the anterior and posterior divisions of the insula, was identified and marked with thread. *b*, A horizontal cut, made through the same specimen, allows a view corresponding to section 6 of the reconstruction program as shown in the top row of Fig. 1. At the same time, a lateral view of the brain and the insular cortex can be seen, with the critical region on the precentral gyrus just anterior to the central sulcus.

the extent of their injuries. If this insular region is indeed reserved for articulatory planning, then overlapping of lesions in this second group of patients without apraxia of speech should demonstrate a sparing of this focal area.

Figure 1*b* shows the overlay of lesions in those patients without apraxia of speech. These lesions span essentially the same arterial distribution as the group of apraxic patients but spare one significant area; the very area which is consistently infarcted in patients with apraxia of speech (Fig. 1*a*, top) is entirely spared in those patients without the disorder (Fig. 1*b*, top).

Thus the role for the insula in articulatory planning is supported by a double dissociation. Every one of the 25 patients with apraxia of speech had a lesion encompassing this discrete region of the insula, whereas none of the lesions in the 19 patients without apraxia of speech involved this critical area. This new area of the brain associated with articulatory planning, as localized on section 6, corresponds to Talaraich coordinates $-41, -2, 10$ (ref. 5).

To determine the exact location of this articulatory planning area, a normal human brain was dissected and comparisons were made with the slices of the reconstruction programme (Fig. 2). Removal of the frontal, parietal and temporal opercula of the brain's lateral surface revealed the island of cortex underneath known as the insula. A small thread was placed in the central sulcus of the insula to mark anterior from posterior insular gyri. Sectioning the brain to compare to the same horizontal slice as the computerized lesion reconstructions revealed that the area of

interest lay on the precentral gyrus of the insula, directly anterior to the central sulcus as marked.

The results are relevant to a number of important issues. First, a specific lesion site for apraxia of speech has never before been described, to my knowledge. This has been due largely to the lack of agreement on terminology and diagnosis as well as inadequate lesion information. Some attempts to localize apraxia of speech have concluded that no specific cortical region controls the planning of articulation⁶. Others have suggested there may be as many as three different types of apraxia of speech, each associated with a different brain area⁷. At one time Broca's area was thought to be involved solely⁸ or in combination with lesions in other areas^{9,10}, but patients with lesions restricted to Broca's area were not found to have persisting apraxia of speech^{11,12}. Kertesz¹³ described ten cases of apraxia of speech with lesions confined to subcortical structures and concluded that these, as well as cortical structures, must be involved in speech praxis. Oral apraxia, a deficit in planning oral movements often found to coexist with apraxia of speech, was related to lesions in the left frontal and central opercula and anterior insula¹⁴. In general, authors have agreed that anterior structures, as opposed to posterior (temporal and parietal) lobes, are typically involved in apraxia of speech (refs 1 and 15, but see ref. 7), though no specific anterior area has been identified.

The second issue is that the functions of the insula, particularly of the precentral gyrus, have been poorly defined. The insula is considered part of the paralimbic cortex¹⁶ and has been implicated in visceral sensory functions (taste, olfactory and gastric sensations), motor functions (respiration and gastrointestinal activity), as a supplementary motor area (gross body movements also including lips, larynx and face) and a vestibular area (receiving projections from the dorsal thalamus)¹⁷. Recent evidence suggests that the insula may also function as a secondary somatosensory area. Recording sites in posterior insular cortex of the rhesus monkey responded to non-noxious somatic, visual, auditory and gustatory stimuli, suggesting an important role for this area in somatosensory processing¹⁸. Others have documented changes in cerebral blood flow to insular areas in response to vibrotactile stimulation of the feet and hands in human subjects¹⁹. Last, cardiovascular responses have been produced with stimulation to the insula in rats and in humans²⁰.

The insula has traditionally not been specified as important in the study of speech and language localization, possibly because of a historical trend which focused instead on other cortical regions for these functions, typically overlooking subcortical structures and insular cortex. One exception is a study²¹ in which electro-

BOX 1 Examples of articulatory errors in patients with apraxia of speech

Patient H.F. (attempting to say 'cushion')
 "Oh, uh, uh, /chookun/ uh, uh, uh, /dook/, I know what it's called, it's c-u, uh, no, it's, it's /chook/ /chookun/, no."
 Patient D.B. (repeating 'catastrophe' 5 times)
 "catastrophe, /patastrofee/, /t/ catastrophe, /katasrifrobee/ aw sh- -, /ka/ /kata/ sh- -, sh- - I don't know."

Patients with apraxia of speech will inconsistently misarticulate words and then struggle for the correct pronunciation in repeated trials. These errors may occur among 'islands of fluent speech'⁸ and appear less often in more automatic utterances such as expletives or common phrases. Changes in rate and prosody also occur, possibly as a result of the difficulty in articulation or as a method of compensation. Because these repetitions resemble the target word, the errors are not considered deficits in word retrieval and patients do not report difficulty in finding the word as much as in producing it. The disorder must also be distinguished from the different types of dysarthrias which are a result of weak or paralysed muscles that actually perform the articulation and are distinguished

TABLE 1 Demographic characteristics of the two patient groups

	Patients with apraxia of speech	Patients without apraxia of speech
Number of patients	25	19
Age	32–79 years	52–80 years
Education	8–18 years	8–18 years
Time after onset of stroke	1–14 years	1–11 years
Coexisting conditions: (per cent of patients in group)		
Bucco-facial apraxia	48%	0%
Limb apraxia	52%	0%
Dysarthria	40%	0%
Broca's aphasia	52%	0%
Global aphasia	12%	0%
Anomic aphasia	28%	26%
Wernicke's aphasia	0%	21%
Conduction aphasia	0%	11%
Unclassifiable aphasia	0%	5%
No aphasia	8%	37%

cortical stimulation to the insula during epilepsy surgery produced word-finding errors. However, identification of any particular region within the insula was not made. Another study²² describes a case with bilateral infarctions of the insula resulting in severe mutism of a month's duration, and a persistent sound-recognition deficit. This patient also had a severe bucco-facial apraxia and decreased fluency several months after onset, although auditory and reading comprehension as well as naming abilities were near perfect. Several authors have mentioned involvement of the insula in disorders such as conduction aphasia²³, and particularly Broca's aphasia, in which articulatory errors are prominent^{11,24–26}. A number of studies have mentioned increases in blood flow to the insula in tasks involving speech (see, for example, ref. 27), although none has identified any specific area within the insula in relation to articulatory planning.

The possibility that the precentral gyrus might serve as a preliminary processor to the initiation of articulatory movements raises questions about the functions of other discrete regions along this gyrus. The possibility that the insula might also subserve other types of movement planning, such as limb praxis or the articulation of sign-language gestures, is currently being explored.

The result reported here is important for models of cerebral localization that strive to localize language functions as well as other areas of cognition. Although complex cognitive behaviours may be more difficult to localize than motor or sensory functions, determination of specific aspects of language and cognition, coupled with the careful delineation of lesion site, can lead to a better understanding of whether or not these functions might be localizable in the brain. This, in turn, gives us more information about the neural mechanisms of language and cognition. □

Methods

Subjects. Forty-four patients met the following criteria. All had suffered a single left-hemisphere cerebrovascular accident (stroke) with no other history of neurological or psychiatric problems. The injuries had occurred at least one year before inclusion in the study to ensure that the observed speech and language deficits had stabilized. All patients had undergone CT or MRI at least 3 weeks (typically 4–5 years) after onset of their injury to guarantee distinct boundaries of the completed stroke. Patients were all right-handed and native English-speaking with normal hearing. Further demographic information is given in Table 1.

Diagnosis of apraxia of speech. This disorder is difficult to separate from the frequently coexisting effects of aphasia and/or dysarthria and must be diagnosed by a speech-language pathologist trained to recognize the difference. To be certain of the presence or absence of speech apraxia in this study, at least two certified speech-language pathologists were asked to make the diagnoses. This was done either by direct administration of the Motor Speech Evaluation²⁸ or by viewing a videotape of its administration and scoring blind to the other clinicians' diagnosis of the patient. Clinicians listened to patients' performance on rapidly alternating articulatory movements, repetitions

of single words differing in articulatory complexity, repetitions of sentences, samples of spontaneous speech elicited by the description of a picture, and readings of a passage of text. Assessments were based on four criteria measuring a pattern of articulatory behaviour as described by Wertz *et al.* (ref. 28, page 81): '(1) effortful, trial-and-error, groping articulatory movements and attempts at self correction, (2) dysprosody unrelieved by extended periods of normal rhythm, stress, and intonation, (3) articulatory inconsistency on repeated productions of the same utterance, (4) obvious difficulty initiating utterances'. Clinicians observed several instances of each of these criteria throughout the examination before making a diagnosis of apraxia of speech. These patients typically substituted one phoneme for another (usually on initial consonants), had greater difficulty with longer words or sentences, and greater difficulty with repetition than with reading aloud. Clinicians also rated the severity of the performance on a scale of 0 to 7, with '0' indicating no apraxia of speech and '7' reflecting a severe disorder. The mean severity rating for the speech apraxic patients in this study was 4.0. Patients diagnosed without apraxia of speech did not meet all of the above four criteria in their pattern of performance, and all received an overall severity rating of 0. Patients whose diagnosis could not be agreed upon were excluded from this study.

Lesion reconstructions. Lesions were reconstructed onto templates derived from the atlas of ref. 29, representing 11 horizontal slices through the brain at an angle of 0° to the orbital–meatal line.

Reconstructions were done by a board-certified neurologist (R.T.K.) who was particularly experienced in CT and MRI and a co-designer of the reconstruction software. Lesions were not simply traced, but reconstructed in relation to neuroanatomical landmarks to compensate for any discrepancies in scanning angles. Both CT and MRI scans were used, as available, from patients' radiology files. In 89% of the cases, CT scans were used, including 23% of all patients who had both CT and MRI scans. MRIs alone were available from the remaining 11%. Lesion boundaries were entered into a PDP 11/45 minicomputer via electronic bitpad and overlays were derived from the reconstruction software⁴. Determinations of the presence or absence of apraxia of speech were made blind to knowledge of lesion localization, just as lesion reconstructions were made blind to the diagnosis of the behavioural deficit.

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Odour encoding by temporal sequences of firing in oscillating neural assemblies

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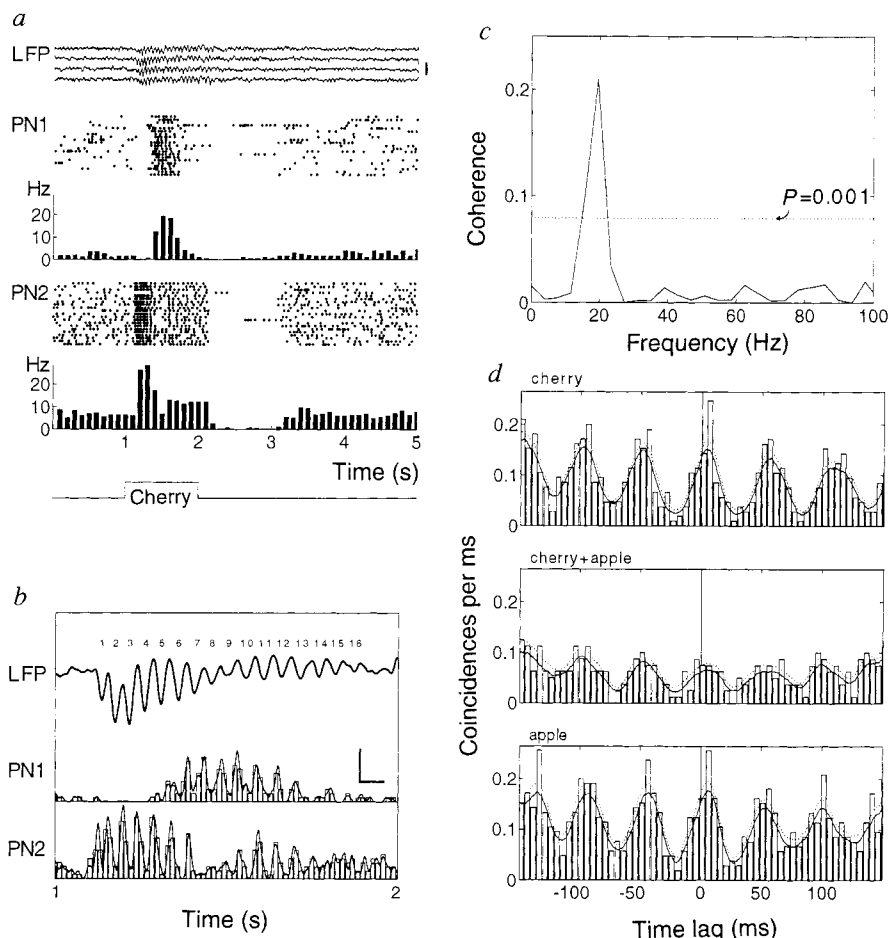
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STIMULUS-EVOKED oscillatory synchronization of activity has been observed in many neural systems, including the cerebral cortex of mammals and the brain of insects¹⁻⁸. The possible functions of such rhythmic synchronization in neural coding, however, remain largely speculative⁹⁻¹³. In the locust, odours evoke activity in dynamic (evolving) ensembles of transiently synchronized neurons^{8,14,15}. We report here that the active neurons composing these ensembles change in a stimulus-specific manner and with a high degree of reliability on a cycle-by-cycle basis during an odour response. Hence, information about an odour is contained not only in the neural assembly active at each oscillation cycle, but also in the precise temporal sequence in which these assemblies are updated during an odour response. Neural coding with oscillations thus allows combinatorial representations in time as well as in space.

Although stimulus-evoked oscillations of brain potentials caused by synchronized neural activity have been known for about 50 years, the roles, if any, they might play in information coding remain conjectural. We tested two functional hypotheses of temporal codes that use oscillations. The first proposes that oscillations, by virtue of their periodic nature, allow the phase of neural signals (that is, the timing of action potentials relative to a specific or common reference during each cycle) to be a coding parameter. Different and possibly coexisting stimuli could thus be represented by different neural assemblies, respectively defined by a common phase of activity^{10,11,13,16,17}. This hypothesis predicts that the neural assemblies activated by different stimuli should synchronize at different phases. The second hypothesis rather proposes that oscillations allow the rank order (for example, cycles 1, 2 and 4 of the oscillation) of action potentials produced by participating neurons to be coding parameters. In this scheme the order of recruitment of neurons in an oscillating assembly should be stimulus-specific.

To test these hypotheses, we used the olfactory antennal lobe of the locust, in which individual odours are represented combinatorially by oscillating and dynamic neural assemblies, each formed by about 10% of a total of about 800 projection neurons (PNs)^{8,14,15}. We recorded simultaneously the responses of small ensembles (2–5) of PNs—the analogues of the mitral-tufted cells of the vertebrate olfactory bulb—*in vivo*, to airborne odourants. Figure 1a illustrates the temporally modulated responses of two PNs to a cherry blend. Although both neurons responded in part

FIG. 1 Odours evoke reliable temporal response patterns in projection neurons, with tight oscillatory synchronization and neuron-specific modulation of periodic firing probability. The phase of firing contains no information. **a**, Rasters and peristimulus histograms (PSTHs) of the responses of two simultaneously recorded antennal lobe PNs to the odour cherry (21 trials at 0.1 Hz, traces aligned on the odour delivery pulse onset). The local field potentials (LFP) recorded in the ipsilateral mushroom body during trials 1–4 are shown. Note that PN1 was briefly inhibited, later excited, and finally inhibited again, whereas PN2 was excited in two successive phases and then inhibited at the offset of the stimulus. Note the duration of the 20-Hz LFP oscillations. Calibration (LFP): 500 μ V. **b**, Fine temporal structure of the PNs' responses during the 1–2 s epoch in **a**. Spikes produced by each PN over the 21 trials were binned in 15-ms bins (histograms) or convolved with a 5-ms-wide gaussian function (solid lines), giving a smooth evolution of the PN firing probability, free of binning artefacts²⁹. LFP trace is average of 21, indicating very tight stimulus-locking with this odour. Note that, when both PNs fired together, their action potentials were synchronous and locked to the LFP oscillations. Calibrations: horizontal, 50 ms; vertical, 30 Hz, or $P = 0.03$ of firing per 1 ms bin. Probability of firing during any period is obtained by integrating the solid line over this period, provided that it does not exceed the shortest interspike interval (which might result in $P > 1$). **c**, Coherence function¹⁸ (varies between 0 and 1) calculated for the spikes produced by these two PNs over the 1-s-long epoch in **b** over all 21 trials. The peak at 20 Hz well exceeds the $P = 0.001$ significance level. **d**, Crosscorrelation functions calculated between the spike trains produced by both PNs in **a** and **b**. Spike coincidences summed, respectively, over 21, 16 and 21 trials for cherry, cherry + apple and apple, were binned in 5-ms bins (histograms) or



convolved with a 5-ms-wide gaussian function (solid lines). Dotted lines indicate one standard deviation of the mean. Note that the roughly 5 ms delay between the two PNs (PN1 leading) is independent of the odour.

TABLE 1 Firing probabilities of neurons in response to odours

Cherry + apple				Geraniol + cherry				
Prior probabilities				Prior probabilities				
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3	Cycle 4
P(PN1)	0.96	0.87	0.39	P(PN1)	0.60	0.50	0	0
P(PN2)	0.04	0.70	0.52	P(PN2)	0	0	0.40	0.30
Joint probabilities: $P(\text{PN1}, \text{PN2})$				Joint probabilities: $P(\text{PN1}, \text{PN2})$				
		PN1				PN1		
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3	Cycle 4
PN2	Cycle 1	0.04	0.04	0	Cycle 1	0	0	0
	Cycle 2	0.65	0.61	0.26	Cycle 2	0	0	0
	Cycle 3	0.48	0.43	0.22	Cycle 3	0.40	0.30	0
				Cycle 4	0.20	0.20	0	0
Conditional probabilities: $P(\text{PN1} \text{PN2})$				Conditional probabilities: $P(\text{PN1} \text{PN2})$				
		PN1				PN1		
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3	Cycle 4
PN2	Cycle 1	1.0	1.0	0	Cycle 1	–	–	–
	Cycle 2	0.94	0.88	0.38	Cycle 2	–	–	–
	Cycle 3	0.92	0.83	0.42	Cycle 3	1.0	0.75	0
				Cycle 4	0.67	0.67	0	0
Conditional probabilities: $P(\text{PN2} \text{PN1})$				Conditional probabilities: $P(\text{PN2} \text{PN1})$				
		PN1				PN1		
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3	Cycle 4
PN2	Cycle 1	0.05	0.05	0	Cycle 1	0	0	–
	Cycle 2	0.68	0.70	0.67	Cycle 2	0	0	–
	Cycle 3	0.50	0.50	0.56	Cycle 3	0.67	0.60	–
				Cycle 4	0.33	0.40	–	–

Action potentials of one PN during a given oscillation cycle have predictive value about the likelihood of firing of another PN during the same or a different cycle. Firing probabilities of the two PNs in Figs 2 and 3 during the first few cycles are shown for cherry + apple (left) and geraniol + cherry (right). The 'prior' probability is the probability that a PN fired an action potential in a given cycle. The 'joint' probability ($P(\text{PN}_x, \text{PN}_y)$) is the probability that PNs x and y fired together during the same trial, with a specific temporal relationship (for example, PN1 in cycle 1 and PN2 in cycle 3). The 'conditional' probability ($P(\text{PN}_y | \text{PN}_x)$) is the probability that PN y fired an action potential during a given cycle, given that PN x fired an action potential in the same, or a different cycle, of the same trial (for example, the conditional probability that PN2 fired an action potential in cycle 3, given that PN1 had fired in cycle 1 of the same trial, was 0.67 in response to geraniol + cherry). See text for details; numbers in bold are referred to in the text. When conditional probabilities so calculated were greater than the corresponding prior probabilities, they exceeded them by an amount varying between 0 and 0.45. The average increase was 0.21 ± 0.085 ($n = 45$).

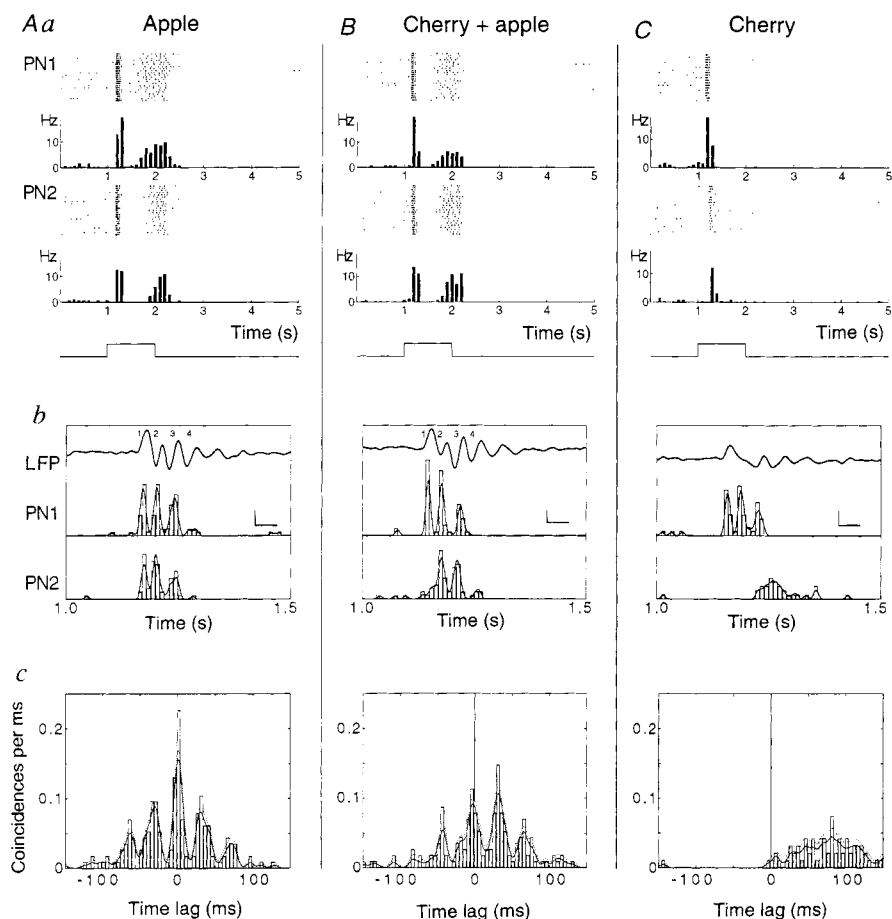
with an increased firing rate, their peaks of activity did not coincide and the durations of the periods of increased firing differed between them. Figure 1b illustrates the fine temporal structure of these responses, revealing that the firing probability of each PN varied rhythmically and that the timing of either neuron's activity was locked to the other's and to the field potential oscillation. The magnitude squared coherence¹⁸ of the activity of these two PNs over the 1-s response period was significantly different from zero ($P < 0.001$) at 20 Hz, the frequency of the field potential oscillations (Fig. 1c), indicating tightly correlated firing. We presented 13 odours and their mixtures. For all odours with which the recorded neurons phase-locked to the field potential, we measured the phase of each neuron relative to the field potential (not shown) or to the other neuron (Fig. 1d), by cross-correlating their responses. Figure 1d, for example, indicates that, although these two neurons fired and phase-locked in response to cherry, apple, or a mixture of both, the slight phase difference between their action potentials was constant and thus not odour specific. This analysis was repeated with five pairs of PNs and 17 'neuron pair-odour' combinations for which coherence values were significant at $P < 0.01$. In no case did the phase of action potentials relative to the field potential or to action potentials of other participating neurons appear to vary with, and thus participate in encoding, the stimulus.

We next considered the second hypothesis. Figure 1b, for example, indicates that, although both neurons were synchronized in response to cherry, coincident firing of both PNs occurred only during certain cycles of the oscillatory ensemble response (for example, cycles 6–7, 10–13), and never during other cycles (for

example, 1–4). This information cannot be extracted by cross-correlation analysis (Fig. 1d), which averages together successive events, thereby obscuring the temporal details of correlated activity. Are such temporal patterns reliable and stimulus-specific? Figure 2Aa, Ba illustrates the gross responses of a different pair of neurons to the odour apple and the mixture cherry + apple. In both cases, the two neurons produced remarkably similar responses: a short 'burst' of action potentials, a period of silence and a second, longer period of elevated activity. These responses (rasters and peristimulus histograms) seemed, at first glance, to be identical for both odours. Consider now their fine temporal structure during the first 500 ms of the response (Fig. 2Ab, Bb). Whereas PNs 1 and 2 both fired reliably during cycles 1–3 in response to apple (Fig. 2Ab), PN2 failed to fire during cycle 1 in response to the mixture, resulting in a relative shift of the two PNs' firing by one cycle (Fig. 2Bb). Such 1- or 2-cycle relative shifts were observed for several other odour combinations to which both neurons responded (Fig. 3). The skewness of the crosscorrelation functions calculated over two PNs' responses to odours could be indicative of the absence (for example, Fig. 2Ac) or presence (for example, Fig. 2Bc) of a sequential activation of the two neurons, but could not reveal the exact temporal structure of these sequences.

These fine temporal patterns were highly reliable. First, the firing probability of a PN during a given cycle could exceed 0.9 (for example, cycle 1 for PN1 in response to cherry + apple; Fig. 3 and Table 1), indicating participation of this PN in the odour-coding assembly during this cycle of almost any trial. Second, the firing of one PN during a given cycle often had some predictive value about

FIG. 2 Stimulus identity is encoded in part by the fine temporal features of firing of groups of neurons. A–C, Responses of two PNs (different animal to that in Fig. 1) to two odours (A, C) and their 1:1 mixture (B). a, Rasters and PSTHs constructed as in Fig. 1a. b, Fine temporal structure of the PNs' responses during the 1–1.5 s epoch (representation as in Fig. 1b, bin size: 10 ms). c, Crosscorrelation functions calculated with the PNs spike trains (as in Fig. 1d). Note the similarity of both neurons' overall responses to apple and cherry + apple (Aa, Ba), but the striking difference between the fine structures of their spike trains (Ab, Bb). Note that the cross-correlation function is symmetrical about the y-axis at $x = 0$ (zero time lag) for apple (indicating near perfect average synchrony), but that it is skewed to the right (meaning that PN1 fired one cycle earlier on average than PN2) for cherry + apple. The exact temporal sequence of activation, however, can only be seen in b. Note, finally, that the response to cherry alone was shorter in both neurons (it lacked the second excitatory epoch, Ca), and that only PN1 showed an oscillatory response to this odour (Cb, c). Calibrations (b): horizontal, 50 ms; vertical, 20 Hz, or $P = 0.02$ of firing per 1 ms bin.



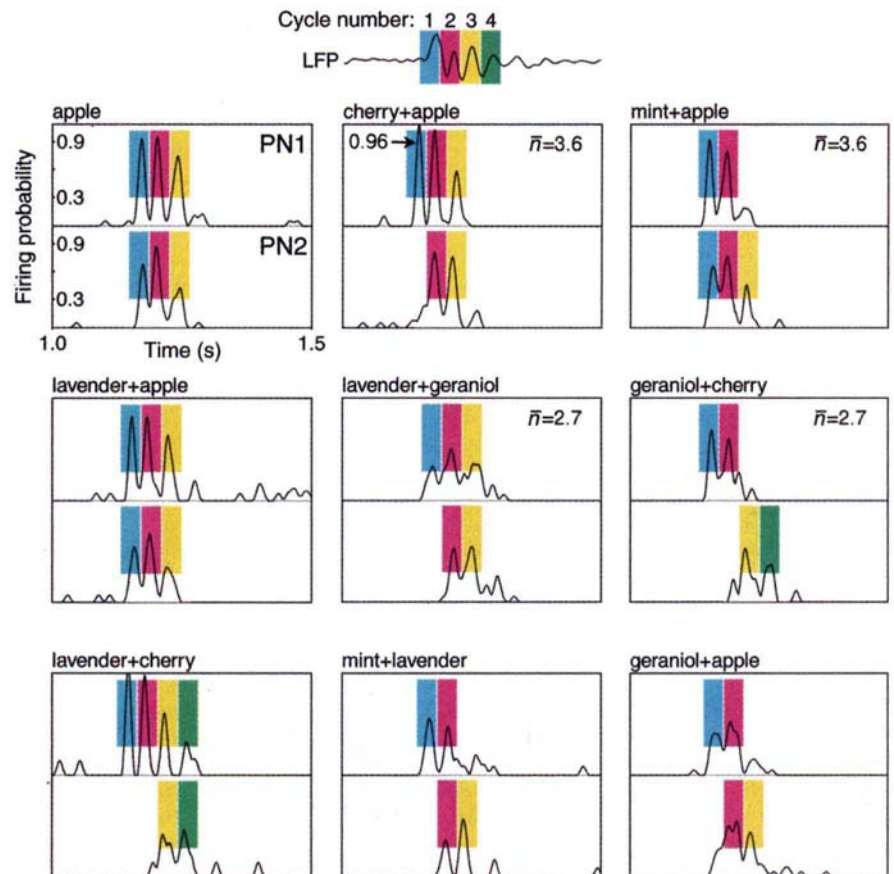
the behaviour of another PN during the same, or a different, cycle. For example, although the firing probability of PN2 at cycle 3 was 0.4 in response to geraniol + cherry, this probability rose to 0.67 if it was known that PN1 had fired at cycle 1 (Table 1). Similarly, whereas the firing probability of PN1 at cycle 1 was 0.6 in response to this odour, it rose to 1.0 if it was known that PN2 would also fire at cycle 3 (Table 1). These results show that odours can reliably evoke specific sequences of activity across neural assemblies.

The fine temporal structure of PN responses to mixtures were not simple or predictable combinations of their responses to the components presented separately (compare Fig. 2A–C). Each odour, however complex, apparently evoked activity in its own specific neuronal set and its own specific sequence of activity (coarse and fine levels of analysis, Fig. 2). For example, unique patterns are discernible in the firing probabilities of the two PNs in Fig. 2 in response to many other odours (Fig. 3). Each oscillation cycle is represented by a different colour, and the existence of a firing probability greater than 0.3 for each PN and cycle is indicated by a coloured box. This representation reveals six different patterns for these nine odours (Fig. 3), indicating that knowing the average temporal firing behaviour of these two neurons over four oscillation cycles provides a 0.66 chance of correct odour identification. Ignoring this temporal information would, by contrast, provide only a 0.33 chance of correct identification (three different spike counts for nine odours), a probability similar to that provided by considering the temporal firing behaviour of one only of these two PNs (3 different temporal patterns for 9 odours in each PN; Fig. 3). In reality, PN firing is probabilistic and average firing probabilities are not available to the animal on a single odour sampling. Information is, therefore, probably gathered from multiple neurons at once as well as over several oscillation cycles. Previous results indicate that odours are

probably represented by about 100 neurons^{8,15}, only a subgroup of these are likely to be coactive during any given oscillation cycle.

In summary, we have shown that odours evoke activity in neural assemblies that are updated non-randomly at each cycle of an oscillatory response pattern. Information about the stimulus is found not in the phase of the active neurons (which is statistically invariant when neurons synchronize to each other) but rather in their identity^{8,14,19,20} and in the temporal pattern in which they are recruited (this study). The firing probability of individual neurons during one cycle of the response can be linked to that of other neurons during the same or other cycles, indicating deterministic and stimulus-specific sequential activation of groups of neurons. In principle, the timing of these odour-encoding sequences need not be precisely locked to the stimulus, although it is this feature which allowed us to detect them. We propose that oscillations are a means to encode stimuli in a temporal combinatorial fashion. In this hypothesis, stimuli are represented both by spatial (instantaneous) ensembles, that is, the neurons activated together during any given cycle, and by temporal sequences, reflected in the responses of individual or groups of neurons over several cycles. Given the probabilistic nature of any neuron firing in any one cycle of the oscillatory response, the information provided by spatial ensembles alone is often ambiguous. We propose that the brain reduces the ambiguity by assigning 'meaning' to the pattern in which these ensembles succeed each other. In particular, such a mechanism might aid reliable and rapid stimulus identification. In mammals, for example, each inspiration evokes 5–10 cycles of gamma frequency (30–60 Hz) oscillations in the olfactory bulb^{21,22}. In insects, behavioural studies indicate that odour identification is possible in a few hundred milliseconds²³, allowing in principle only 4–6 cycles of 20–30 Hz oscillations¹⁵. Such a coding mechanism might also occur in any sensory system (cortical or not) expressing

FIG. 3 Information about an odour can be gained from the temporal structure of firing of the neurons activated in periodic synchrony. The temporal evolution of the firing probabilities of the two PN in Fig. 2 in response to nine complex odours is displayed here. If the firing probability of either PN exceeded a given threshold ($P = 0.3$) during one oscillation cycle of the response, the neuron's action potential was assigned a colour corresponding to the cycle in which it occurred. A coloured box thus indicates that the corresponding PN fired an action potential during that cycle with a probability $P > 0.3$ (integrated over the 20 ms period centred on the peak firing probability). The average LFP in response to apple is shown at the top, with each oscillation cycle denoted by its colour. Each odour can thus be represented by a particular coloured pattern that contains more information about odour identity than spike count alone. For example, whereas cherry + apple and mint + apple each produced an average of 3.6 action potentials per trial in the two PN over the first 3 cycles, the two odours could be discriminated because these action potentials occurred in a different order with each odour. Similarly, lavender + geraniol and geraniol + cherry each produced an average of 2.7 action potentials per trial in the two PN over the first four cycles, but the two odours could be discriminated by the sequence of these action potentials.



oscillatory synchronization, such as the visual^{13,17} and somatosensory²⁴ cortices of mammals. Evidence from flies and from rat, cat and primate neocortical neurons *in vitro*²⁵ and *in vivo*^{26,27,28} is also consistent with the possibility that spike timing might play a role in information coding there, although these studies did not consider spike timing within the context of oscillatory synchronization. This study shows that oscillations can support combinatorial coding in time, and that information about a stimulus is contained in the precise temporal firing pattern of groups of neurons. □

Methods

Forty adult locusts (*Schistocerca americana*) of both sexes taken from a crowded colony were immobilized with one antenna intact, as in ref. 14. A window was cut open on the head and the brain was gently desheathed and intermittently superfused with locust saline (in mM: 140 NaCl, 5 KCl, 5 CaCl₂, 4 NaHCO₃, 1 MgCl₂, 6.3 HEPES, pH 7.0).

Odour stimulation. Airborne odour puffs (1 s long, 0.3 l min⁻¹) were delivered from a circular array of stainless steel nozzles placed 21 mm in front of the antenna. All nozzles were angled so as to converge on the antenna. Clean, dry air was constantly delivered at a rate of 0.3 l min⁻¹. Each odour (10 µl of apple, vanilla, strawberry (Gilberties, Easton, CT), cherry (Bell Flavors and Fragrances Inc.), spearmint (Flavco, Mansfield, OH), lavender (Nuit Unlimited), eugenol, geraniol (Sigma), cineole, isoamylacetate, citral (Aldrich), crushed banana, alfalfa chow) was carried by a distinct nozzle to exclude crosscontamination along the odour path. Mixtures were applied by simultaneously delivering selected pairs of odours, each at the rate of 0.3 l min⁻¹.

Electrophysiology. Each PN (up to five simultaneous recordings) and the local field potential (LFP) were recorded extracellularly with blunt glass micropipettes (horizontal puller; Sutter, Novato, CA) filled with saline, and amplified with DC amplifiers (Axon, Foster City, CA; NPI, Adams-List). Traces were stored on digital tape (Micro Data Inc., 5.5 kHz cut-off) and analysed off-line, using National Instruments NBM16L hardware and LabVIEW (National Instruments) and MatLab (The MathWorks) software. PN action potential times were obtained by applying a digital threshold discriminator algorithm to traces containing a singular action potential waveform. PN isolation was thus unambiguous. LFP traces were low pass filtered at 50 Hz (digital non-causal 5-pole Butterworth). The data presented come from 200 neuron pairs. Of these, 45 pair-odour

combinations showed sufficiently long epochs of coincident firing of the two neurons. These were analysed exhaustively, providing 17 (from 5 different neuron pairs) with a coherence¹⁸ at 20–30 Hz that was statistically significant at $P < 0.01$. Given that individual odours probably evoke synchronized activity in about 10% of the 830 PN⁸, the chance of recording simultaneously from 2 neurons that synchronize in response to the same odour should be about 1% of the obtained pairs. Our results are in good agreement with these estimates.

Statistics. Prior and joint probabilities were computed by counting the mean number of action potentials (prior) or coincidences (joint) in 10 ms epochs centred on the peaks of the firing probability curve in each cycle (peaks of solid lines in Figs 2b and 3). Conditional probabilities are the quotient of the joint and prior probabilities. Because the PN⁸ never fired more than once during any cycle, normalization was not necessary and these values varied between 0 and 1. Because conditional probabilities are ratios, they are undefined (denoted by a dash in Table 1) when the prior probability in the denominator is zero.

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Spatio-temporal dynamics of cyclic AMP signals in an intact neural circuit

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THE functional properties of neuronal networks can be reconfigured by a variety of modulatory neurotransmitters, which may alter the excitable properties of neurons or the strengths of synaptic connections. Many of these neuromodulators act via the intracellular second messenger cyclic AMP, but their effects on the spatial distribution of cAMP concentration have never been examined in an intact neural circuit. We therefore used the cAMP-indicator dye FICRhr (refs 1, 2) to investigate the effect of several neuromodulators (octopamine, dopamine, acetylcholine, serotonin and proctolin) on cAMP distribution in identified neurons of the lobster stomatogastric ganglion (STG). When added to the bath solution, each of these neuromodulators produced a unique pattern of cAMP transients among the different neurons of the STG. Electrical stimulation of neurons innervating the STG causes synaptic release of endogenous modulators, leading within a few seconds to local increases of cAMP in fine neurite branches, the site where many modulators are thought to act^{3,4}. After prolonged stimulation, cAMP diffuses from the site of production to throughout the neuritic tree and eventually to the cell body. Diffusion of cAMP may explain how transient localized inputs to a neuron can produce long-range effects such as long-term changes in gene expression.

The stomatogastric ganglion of the spiny lobster *Panulirus interruptus* comprises about 24 interconnected motor neurons and 6 interneurons arranged into two central pattern generators that control the striated muscles of the stomach⁵. The neural circuitry of the STG can be reconfigured by any of the approximately twenty neuromodulators that are known to be released onto the ganglion by afferent fibres in the stomatogastric nerve (STN)⁶. The actions of at least some of these modulators are believed to be mediated by cAMP⁷. We have used cAMP imaging in the STG to examine the signal transduction events that underlie circuit reconfiguration.

The stomatogastric nervous system was removed from adult lobsters using a standard dissection procedure⁸ that leaves the neural circuitry of the STG and its modulatory inputs intact. After electrophysiological identification, STG neurons were pressure-injected with fluorescein- and rhodamine-labelled cAMP-dependent protein kinase (PKA) type II (FICRhr; refs 1, 2) to image cAMP concentration ([cAMP]) by high-speed confocal microscopy⁹ (Fig. 1a). The ratio of fluorescein to rhodamine emissions from FICRhr is sensitive to cAMP concentrations over the range 0.05–10 μ M at the ionic strength of marine invertebrate cytoplasm¹⁰. Unfortunately in this preparation it

was difficult to use cAMP antagonists to impose an effectively zero cAMP concentration [cAMP] in order to determine the minimum fluorescence ratio and so calibrate FICRhr signals in terms of absolute [cAMP]. Therefore, our main conclusions rely only on relative changes in [cAMP] over time. Application of the adenylyl cyclase activator forskolin to the STG increased the ratio to a level that could not be raised further by the phosphodiesterase inhibitor IBMX and the membrane-permeable PKA activator S_p -5,6-Cl₂-cBIMPS, showing that forskolin increased [cAMP] enough to saturate the FICRhr ratio change. All the responses to neuromodulators described showed a ratio change smaller than the saturating forskolin response, that is, less than 10 μ M free cAMP.

Bath application of several modulators known to be present in STN fibres produced transient increases of somatic [cAMP]. These transients were cell-specific, that is, each modulator produced a distinct pattern of responses across the ganglion (Fig. 1b–d; Table 1). The patterns of [cAMP] transients were accompanied by well known changes in the electrophysiological output of the circuit^{11–14}, which did not seem to be affected by FICRhr or by imaging (Fig. 2). As cAMP has powerful effects on the electrophysiological activity of STG neurons⁷, the observed pattern of cAMP responses must contribute substantially to the electrophysiological changes. However, comparisons of the patterns of [cAMP] changes with the cell-specific patterns of electrophysiological effects of each modulator did not show any simple correspondence between the two, indicating that the relationship is complex, and probably involves other second messenger pathways.

To determine the effect of synaptic release of endogenous neuromodulators on [cAMP] in postsynaptic cells, we directly stimulated neuromodulatory afferent fibres in the STN. Extracellular STN stimulation lasting 2–3 min elicited [cAMP] transients in the somata of many STG neurons (Fig. 2a, b). However, the latencies of somatic [cAMP] transients induced by STN stimulation were far too long to account for the rapid electrophysiological effects that occur within seconds of STN stimulation^{15,16}. This suggested that responses would be faster in other regions of the cell. STG neurons are complex structures with an extensive neuritic arbor which could be imaged in cells well filled with FICRhr (Fig. 3a). The fastest [cAMP] increases were seen in the finest neurites (2–3 μ m diameter) that we were able to image (for example, see Fig. 3c). As little as one second of STN stimulation was sufficient to produce a detectable [cAMP] increase in these fine neurites. Larger neurites did not respond at all to such short stimuli. Response latencies were as low as 3 s, with a mean of 6.6 s for the most rapidly responding neurites in five separate neurite fields from three preparations. We expect that the response kinetics of the finest neurites in the ganglion (less than 1 μ m diameter), which were below our threshold for detection, would be fast enough to account for the electrophysiological changes that can be brought about in just a few seconds by STN stimulation¹⁶. The location of the rapid transients is also appropriate, because the synaptic structures³, and probably some of the ionic conductances⁴ that are the targets of neuromodulation, are located preferentially on neuritic branches of these neurons.

Imaging neurites during longer periods of STN stimulation revealed a centripetal pattern of [cAMP] increases, with finer neurites responding most rapidly (24 ± 4 s to 50% of maximum response; $n = 20$, mean $\pm$ s.e.m.), then primary neurites (53 ± 6 s; $n = 20$) and finally somata (245 ± 35 s; $n = 15$) (Fig. 3b, c). The difference in response times between higher-order neurites and primary neurites and between primary neurites and somata were both statistically significant (Student's *t*-test, $P < 0.01$).

As cAMP is produced at the membrane by adenylyl cyclase and subsequently diffuses into the cytosol, the faster [cAMP] elevations in smaller neurites could be solely a result of their larger surface-area-to-volume ratio¹⁷. Three observations are inconsistent with this hypothesis. First, STN-induced [cAMP] elevations in larger neurites and somata were delayed relative to finer neurites

not only in the time taken to reach 50% response but also in onset latency (Fig. 3c). Surface-area-to-volume ratio differences could explain differences in peak response latency but not differences in onset latency. Second, in favourable situations, we observed [cAMP] elevations travelling unidirectionally through long, uniform-diameter segments of neurites (Fig. 3b, c). Third, somata responded much earlier to bath-applied amines (onset

latency, 21 ± 2 s) than to STN stimulation (93 ± 22 s; difference is statistically significant: $P < 0.01$, Student's *t*-test), showing that the limiting factor in the speed of somatic response to STN stimulation is not surface-area-to-volume ratio, but rather some other phenomenon such as diffusion of cAMP from remote neurites.

The pattern of cAMP spread could also be explained by

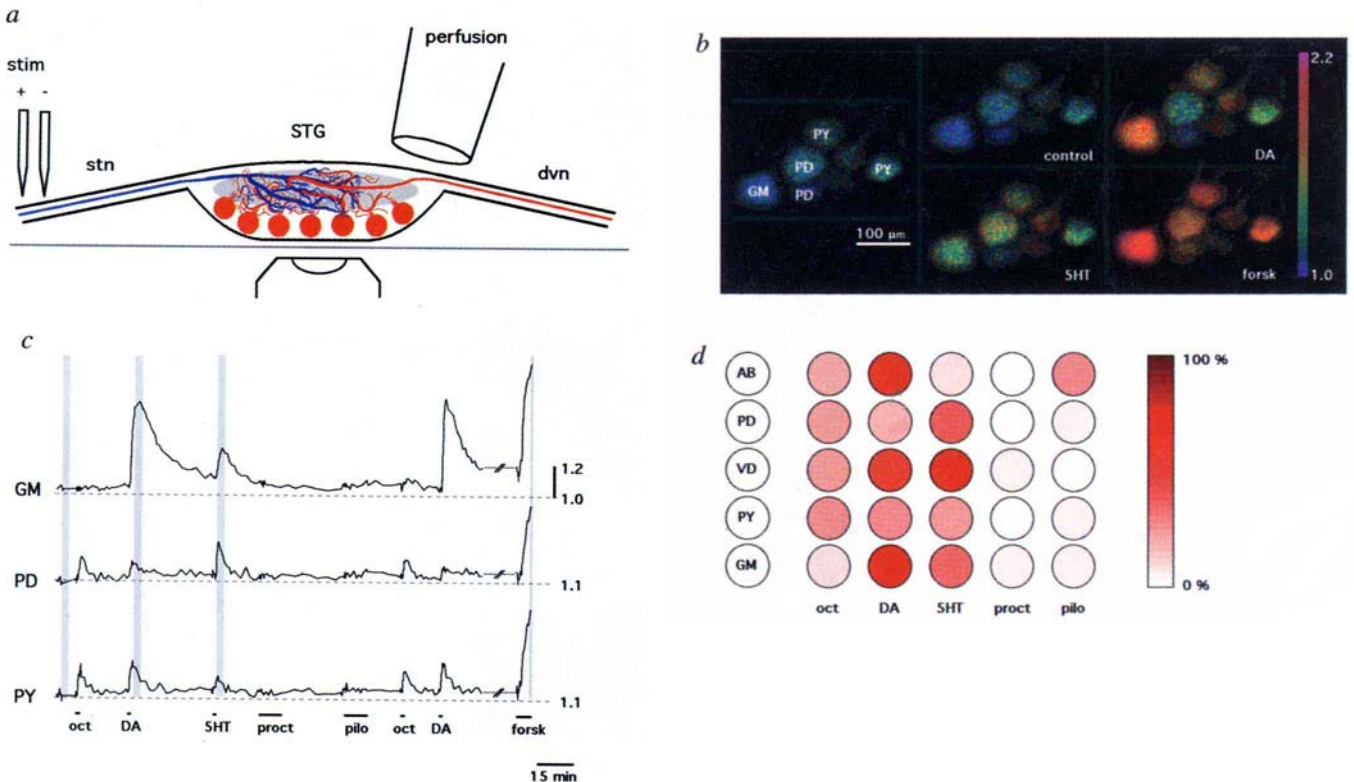


FIG. 1 Different neuromodulators produce distinct patterns of cAMP responses in the STG. *a*, The preparation. Activity of STG neurons (red), whose motor axons leave the DVN, is influenced by neuromodulatory fibres (blue) that originate in higher ganglia, project through the STN and terminate in the STG neuropil (grey). STG, stomatogastric ganglion; STN, stomatogastric nerve; DVN, dorsal ventricular nerve. *b*, Fluorescence ratio images of part of a stomatogastric ganglion in the presence of agonists DA, 5HT and forskolin. Left-hand panel gives a key to different cell types; unnamed cells are unidentified. Scale is in arbitrary units of fluorescein: rhodamine fluorescence ratio². *c*, Data from *b* expressed as average fluorescence ratio over the entire somata of the selected cells. Grey vertical bars indicate the time over which samples were averaged to

generate the corresponding images in *b*. Black horizontal bars indicate the times of agonist application; the black vertical bar associated with the top trace shows the fluorescence ratio scale for all traces. The offset of each trace is shown by the associated dotted line. Although the forskolin response does not appear to reach a plateau here, forskolin was applied for a time known from previous experiments to be sufficient for a maximal response. *d*, Summary of modulator-induced fluorescence ratio changes in STG neurons. Shading of each cell in the array indicates the peak ratio change induced by the modulator listed, expressed as a percentage of the ratio change induced in that cell by forskolin. The data shown are presented in Table 1. Abbreviations are given in Methods.

TABLE 1 Summary of modulator induced somatic fluorescence ratio changes

Cell type	Baseline	Forskolin	Octopamine	Dopamine	Serotonin	Proctolin	Pilocarpine	n1	n2	ANOVA
AB	1.28 ± 0.04	1.62 ± 0.17	19 ± 5	63 ± 21	9 ± 3	-2 ± 8	25 ± 26	3	3	s
PD	1.19 ± 0.12	1.61 ± 0.13	23 ± 10	17 ± 7	38 ± 14	2 ± 3	6 ± 21	6	2	n
VD	1.12 ± 0.17	1.49 ± 0.24	23 ± 16	52 ± 10	64 ± 15	3 ± 20	-3 ± 0	4	2	s
PY	1.07 ± 0.08	1.53 ± 0.17	26 ± 15	28 ± 10	24 ± 13	2 ± 3	6 ± 7	7	4	n
GM	1.19 ± 0.12	1.70 ± 0.06	8 ± 5	76 ± 15	37 ± 11	4 ± 2	3 ± 9	4	3	s
ANOVA	n	n	n	s	s	-	-			

Each value represents the mean peak ratio change elicited by the given agonist in the given cell type. For baseline values and forskolin responses, values represent absolute ratio change; for modulators, values are expressed as a percentage of each cell's response to forskolin. Each value is mean ± standard deviation. n1 is n for octopamine, dopamine and serotonin; n2 is n for proctolin and pilocarpine. Results of analysis of variance (ANOVA) tests are listed for each row and column. For each column, an 's' denotes a significance difference between the mean responses of the cell types listed for the condition in question ($P < 0.01$); 'n' denotes no significant difference. For each row, an 's' denotes a significant difference between modulators for the cell type in question. For comparison of agonists, only octopamine, dopamine and serotonin data are included; all cell types show significant differences between modulators if either forskolin or proctolin and pilocarpine are included. Proctoline and pilocarpine were not tested for differences between cell types because n values were less than 3 for a few cells.

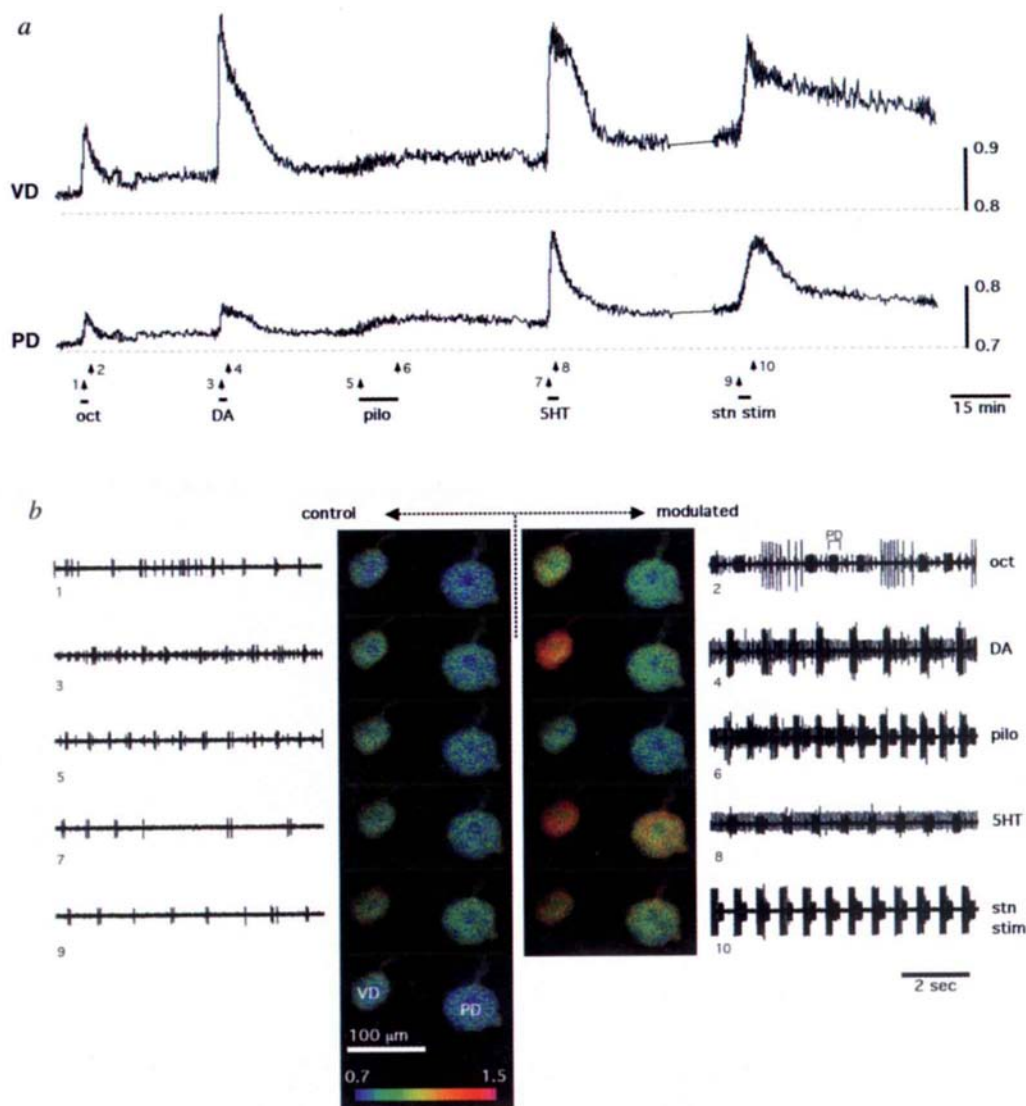


FIG. 2 STN stimulation and bath application of neuromodulators both induce an increase in somatic cAMP concentration and simultaneous changes in electrophysiological activity. *a*, Somatic fluorescence ratio traces from a VD and a PD cell during application of various neuromodulatory stimuli (bars), including STN stimulation. Abbreviations are given in Methods. *b*, Extracellular recordings from the lateral ventricular nerve (LVN) in control conditions (left) and under modulated conditions (right, labelled). These ten samples were acquired at the times indicated by

the arrows in *a*: lower arrows indicate control recordings; upper arrows indicate modulated recordings. This nerve contains axons of the LP cell (largest unit) and the PD cell (smaller unit firing in regular bursts in all modulated conditions except DA), as well as several other units. Ratio images acquired during each of these recordings are paired with the corresponding trace. Bottom panel shows the identity of the two cells and calibration bars. Baseline fluorescence ratios differ from those in Fig. 1 because a different batch of FICRHR was used.

diffusion of neuromodulators, rather than by diffusion of cAMP. Upon longer STN stimulation, a gradient of neuromodulator concentration might be established across the ganglion, from the synaptic neuropil outwards towards the somata. We consider it unlikely that the amount of neuromodulator contained in synaptic terminals is sufficient to bathe the entire ganglion. Furthermore, this hypothesis predicts that adjacent neurites would be exposed to the same concentration of neuromodulators and therefore have the same response latency, but we observed long delays between large and small neurites, regardless of their proximity (for example, IC and PY neurites; Fig. 3*b*). Thus, the only remaining explanation for our results is that cAMP is actually produced primarily in fine neurites and diffuses retrogradely through the arbor and back to the soma.

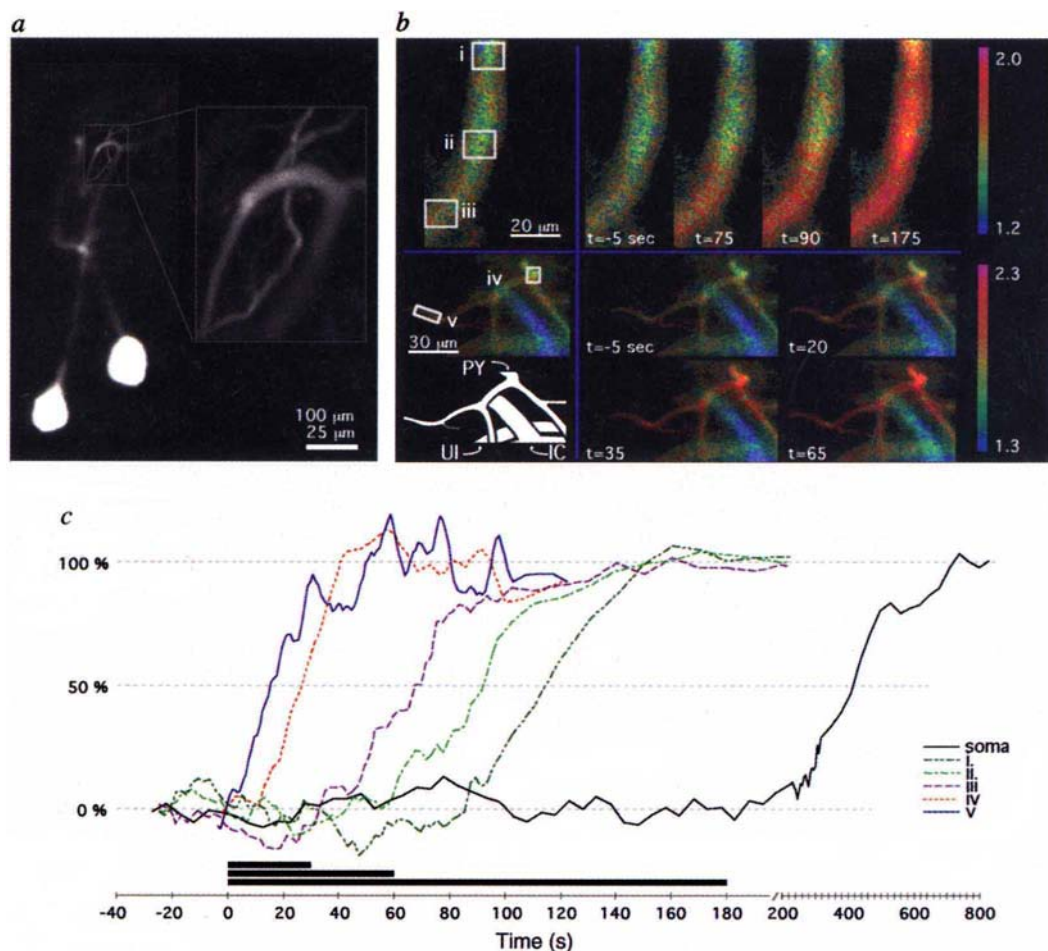
We have shown that, in response to synaptic release of neuromodulators, the neurites of STG cells receive a cAMP signal fast enough to produce rapid reconfiguration of the neural circuitry. The diffusion of cAMP from its site of production provides a

mechanism whereby transient, localized neuromodulatory signals could coordinate functions in physically separate regions of the neuron, such as synapses on different neurite branches. Further diffusion of cAMP into cell bodies has important biological implications because increases in perinuclear cAMP are known to affect gene expression in many systems^{18,19}, which can ultimately effect long-term modification of neuronal properties²⁰. □

Methods

STG neurons were identified under a dissecting microscope using intracellular recordings⁹, and then pressure injected with a solution containing ~3–7 mg ml⁻¹ FICRHR in a solution of (mM): 25 potassium phosphate, 0.5 β-mercaptoethanol, 1 EDTA (pH = 7.2). After injection, the preparation was transferred to a confocal imaging system and pinned in a sylgard/glass chamber. The STN was cut about 2 cm from the STG, which lay dorsal-side-down on a section of coverslip glass to be imaged with the inverted microscope (Fig. 1*a*). Cells could be easily re-identified by comparing fluorescence and transmitted light images to a map of the ganglion drawn on the dissecting microscope. Confocal emission images were simultaneously acquired at two wavelengths⁹ to

FIG. 3 Synaptically released neuromodulators produce local increases in cAMP in fine neurites, which can spread into larger neurites and finally into the soma. **a**, Single wave-length fluorescence image of two FICRhR-filled GM neurons through a $\times 10$ objective. Somata, primary neurites and some secondary neurites are clearly visible. Inset: shows maximum signal and resolution attainable through a $\times 40$ objective. Neurites as fine as $2\mu\text{m}$ in diameter are bright enough to yield useful ratio data. Scale bar: $100\mu\text{m}$; $25\mu\text{m}$ for inset. **b**, Top half: fluorescence ratio image of a portion of the primary neurite of a PY cell, shown at various times during a stimulus trial ($t = 0$ at stimulus onset). The [cAMP] increase is not uniform, but rather proceeds directionally from the distal end of the neurite towards the soma, which is just outside the top of this field. Left panel, regions of interest (i–v) for traces in **c**. Bottom half: a field of fine neurites shown at various times during a stimulus trial ($t = 0$ at stimulus onset). The field includes fine neurites of the same PY cell whose primary neurite is shown in the upper panels. The response of the fine PY neurites is fast in comparison to that of the primary neurite. Also visible in this field, and drawn schematically in the lower left panel, is the primary neurite of IC, together with part of its soma and a segment of primary neurite of an unidentified cell (UI). **c**, Fluorescence ratios from the regions of interest (i–v) shown in **b**, and from the soma of the same PY cell. Comparison of response time courses demonstrates that the most distal neurite (v) of the PY cell responded most quickly, followed by the more proximal fine neurite (iv), then by the primary neurite (iii, ii and i), and finally by the soma. Each trace



was normalized to facilitate comparison of the rise times between the traces (that is, vertical scale represents per cent of maximum response). Black horizontal bars mark the time of STN stimulation (upper bar for (iv) and (v); middle bar for traces (i)–(iii); lower bar for soma); although the stimuli differed in duration, the intensity was identical. This plot represents three separate stimulus trials as it was not possible to monitor all of the regions in a single field of view.

monitor fluorescence in the neurons containing FICRhR. In the figures the logarithmic pseudocolour scale represents the ratio of fluorescein to rhodamine emissions from FICRhR, with higher ratios corresponding to higher concentrations of cAMP^{1,2}. The brightness of each pixel represents fluorescence intensity. Neuromodulators were delivered by a fast, focal perfusion system (wash-on time less than 1 s, wash-off time less than 2 min): octopamine (oct), dopamine (DA) and the muscarinic agonist pilocarpine (pilo) were each $100\mu\text{M}$; serotonin (5HT) and the peptide proctolin (proct) were each $10\mu\text{M}$. The three amines were applied for ~ 1 min, as longer applications did not increase the peak response amplitude (data not shown). Proctolin and pilocarpine were each applied for

10 min because they act more slowly than the amines. Forskolin (forsk, $25\mu\text{M}$) was applied directly to the entire bath to avoid contamination of the perfusion system. Cells in which forskolin failed to evoke a ratio change of at least 20% were considered as unhealthy and therefore excluded. Extracellular recordings were made with bipolar stainless steel electrodes placed in vaseline wells surrounding the lateral ventricular nerve (LVN). The STN was stimulated by using similar electrodes, using 1-s, 50-Hz trains of 0.1-ms pulses delivered at a frequency of 0.5 Hz for 3 min. Cell types are designated throughout the text as follows: AB, anterior burster; PD, pyloric dilator; VD, ventricular dilator; PY, pyloric; GM, gastric mill.

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Timing of neurotransmission at fast synapses in the mammalian brain

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UNDERSTANDING the factors controlling synaptic delays has broad implications. On a systems level, the speed of synaptic transmission limits the communication rate between neurons and strongly influences local circuit dynamics^{1,2}. On a molecular level, the delay from presynaptic calcium entry to postsynaptic responses constrains the molecular mechanism of vesicle fusion³. Previously it has not been possible to elucidate the determinants of synaptic delays in the mammalian central nervous system, where presynaptic terminals are small and difficult to study. We have developed a new approach to study timing at rat cerebellar synapses: we used optical techniques to measure voltage and calcium current simultaneously from presynaptic boutons while monitoring postsynaptic currents electrically⁴⁻⁶. Here we report that the classic view that vesicle release is driven by calcium entry during action-potential repolarization⁷ holds for these synapses at room temperature, but not at physiological temperatures, where postsynaptic responses commence just 150 μ s after the start of the presynaptic action potential. This brisk communication is a consequence of rapid calcium-channel kinetics, which allow significant calcium entry during the upstroke of the presynaptic action potential, and extremely fast calcium-driven vesicle fusion, which lags behind calcium influx by 60 μ s.

A complex sequence of events occurs following action-potential invasion of a presynaptic terminal: voltage-gated calcium channels open, and calcium enters and triggers vesicle fusion, releasing neurotransmitter and activating ligand-gated channels on the postsynaptic cell. Most of what is known regarding the timing of these events is based on the squid giant synapse⁸⁻¹⁰, where the bulk of calcium entry occurs as the driving force for calcium increases during action-potential repolarization; it is this calcium 'tail' current that drives release. At this synapse, the delay to calcium entry and the time required for calcium to trigger vesicle fusion are the primary determinants of the millisecond latency between the onset of presynaptic depolarization and a postsynaptic response^{8,9}.

In the mammalian brain, extracellular recordings suggest that at physiological temperatures synaptic delays are only a few hundred microseconds^{11,12}, but how such short delays are achieved is

unclear. We chose to study synaptic timing in the mammalian brain at the connection between granule cells and stellate cells in cerebellar slices. Stellate cells are spatially and electrically compact, and serve as ideal detectors of neurotransmitter release¹³. In addition, optical techniques can be readily applied to the granule cell parallel fibres, which consist of closely spaced presynaptic boutons of less than 1 μ m in diameter separated by thin axonal segments¹⁴.

Modelling and theoretical analyses have shown that, under certain conditions, the derivative of the fluorescence signals from calcium-sensitive dyes provides a good estimate of the time course of presynaptic calcium currents^{15,16}. This method requires that calcium equilibrates rapidly with the indicator, and that the indicator responds linearly to changes in calcium. We have previously shown that the low-affinity dye mag-fura-5 (refs 17, 18) satisfied these criteria⁶. The time course of the derivative of mag-fura-5 fluorescence changes produced by single stimuli of the parallel fibres (Fig. 1) is similar to that of calcium currents measured electrically in response to action potential voltage clamp^{19,20}, and appears to track calcium influx faithfully. Calcium currents determined with a higher affinity dye, magnesium green^{18,21}, were indistinguishable in time course, indicating that dye kinetics did not distort the calculated calcium currents. The higher-affinity calcium indicator, fura-2 (ref. 22), did not accurately report calcium currents because of its slow kinetics. In all subsequent experiments, calcium currents were determined optically with magnesium green, which provides the best signal-to-noise ratio.

We examined the timing of synaptic transmission by simultaneously measuring presynaptic waveform, presynaptic calcium current, and postsynaptic currents (Fig. 2). Calcium entry starts near the peak of the action potential, about 1.1 ms after action-potential invasion, and reaches a maximum as the action potential repolarizes (Fig. 2a). Postsynaptic currents commence 1.4 ms after the beginning of calcium entry. These two components of the synaptic delay were sensitive to changes in the time course of the presynaptic action potential and the magnitude of the calcium current. This was demonstrated by two manipulations that produce similar synaptic enhancement through different mechanisms (Fig. 2b, c). Elevating external calcium concentration increased the magnitude of calcium currents and the excitatory postsynaptic current (EPSC) without changing the action-potential shape or the calcium-current time course. Slowing the repolarization phase of the action potential by blocking some of the presynaptic potassium channels with tetraethylammonium (TEA) also increased the EPSC, but did so by prolonging the calcium current without significantly altering its peak amplitude. In addition, spike broadening greatly increased the synaptic delay, whereas high external calcium slightly reduced it; understanding these interesting complexities in the kinetics of release will require further

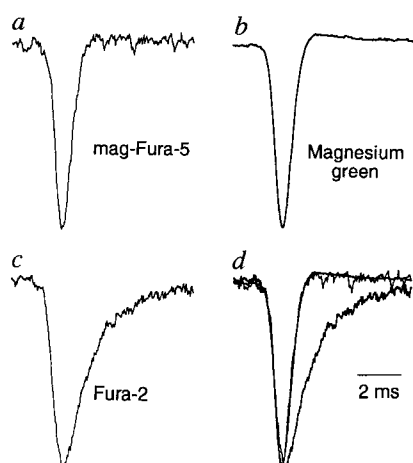


FIG. 1 Derivatives of stimulus-evoked fluorescence transients in parallel fibres. Fibres were labelled with the calcium-sensitive indicators mag-fura-5 (a), magnesium green (b), and fura-2 (c). In d, the traces are superimposed for the purpose of comparison. Traces have been normalized, and are the averages of 4, 3 and 7 experiments, respectively. The half-widths of the derivatives of the fluorescence transients were $790 \pm 40 \mu$ s ($\pm$ s.e.m., range 700 to 870 μ s, $n = 4$) for mag-fura-5, $830 \pm 20 \mu$ s (range 720 to 940 μ s, $n = 11$) for magnesium green, and $1,420 \pm 210 \mu$ s (range 750 to 2,020 μ s, $n = 7$) for fura-2. The range of peak signal sizes ($\% \Delta F/F$ ms⁻¹) was 0.5–1 for mag-fura-5, 6–12 for magnesium green, and 4–12 for fura-2. Temperature = 23 °C.

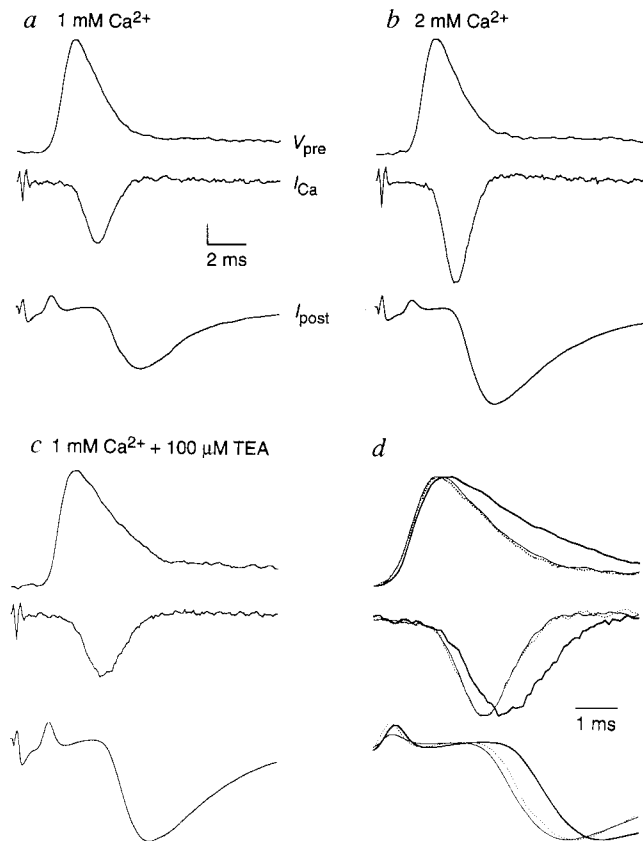


FIG. 2 Timing of presynaptic voltage waveform, presynaptic calcium current, and postsynaptic currents. Simultaneous measurement of presynaptic voltage waveform, presynaptic calcium current, and postsynaptic currents in 1 mM external Ca^{2+} (a), 2 mM external Ca^{2+} (b), and 1 mM Ca^{2+} plus 100 μ M TEA (c). The scale bar in a applies to b and c as well. In d, traces from a (dotted trace), b (thin solid), and c (thick solid), are superimposed and normalized. The vertical scale bar corresponds to 0.02% $\Delta T/T$ for RH482, 1% $\Delta F/F$ ms $^{-1}$ for magnesium green, and 46 pA for the EPSC. Raising external calcium decreased the latency to EPSC by 150 μ s whereas adding TEA increased the delay by 600 μ s. Temperature, 18 $^{\circ}$ C.

studies. This experiment also illustrates our ability to resolve changes in the magnitude and timing of the presynaptic waveform, calcium current, and postsynaptic response.

In these experiments the majority of calcium entry occurs during action-potential repolarization, and the total delay between action-potential invasion and postsynaptic response of 2.5 ms is comparable to the 1.2 ms seen at the squid giant synapse⁸. Thus it appears that the basic biophysical scheme put forward to account for synaptic delays in squid also applies at the parallel fibre to stellate cell synapse at 18 $^{\circ}$ C; however, as we will demonstrate, this does not appear to hold at physiological temperatures.

Increasing the temperature shortened the duration of presynaptic action potentials and calcium currents and reduced the latency to calcium entry (Fig. 3). More significantly, as the temperature was raised there was a gradual transition from almost all of the calcium entering during the repolarizing phase of the action potential to significant calcium entry occurring during the upstroke of the action potential. This change in the nature of the calcium signal that drives release can be explained by two factors: first, calcium-channel kinetics are highly temperature dependent^{23–25}; and second, there is a decrease in spike amplitude at elevated temperatures which increases the driving force for calcium influx at the peak of the action potential²⁶.

We next assessed the delay between presynaptic calcium entry and the EPSC and found that it was highly temperature dependent (Fig. 4). Even at high temperatures, diffusional processes, which are only slightly temperature-dependent, do not appear to be rate limiting. At 14 $^{\circ}$ C the EPSC does not start until the presynaptic calcium current is nearly over, whereas by 38 $^{\circ}$ C, the rising phase of the EPSC lags behind the calcium current by just 60 μ s. This compares to a delay of 1.5 ms for goldfish bipolar neurons²⁷ and 375 μ s for squid giant synapse under action-potential clamp (or 200 μ s when release is driven with a voltage step from +70 mV to –70 mV)⁸.

Thus we have found that the total synaptic delay at the granule cell to stellate cell synapse is 150 μ s at 38 $^{\circ}$ C, allowing local disynaptic computations to be performed in the submillisecond timescale. To achieve such fast transmission, significant calcium influx occurs during the upstroke of the action potential, reducing the delay between depolarization and calcium entry to 90 μ s. In addition, the short latency from presynaptic calcium influx to postsynaptic currents observed at 38 $^{\circ}$ C indicates that the molecular mechanisms responsible for calcium-triggered exocytosis are extremely fast. $\square$

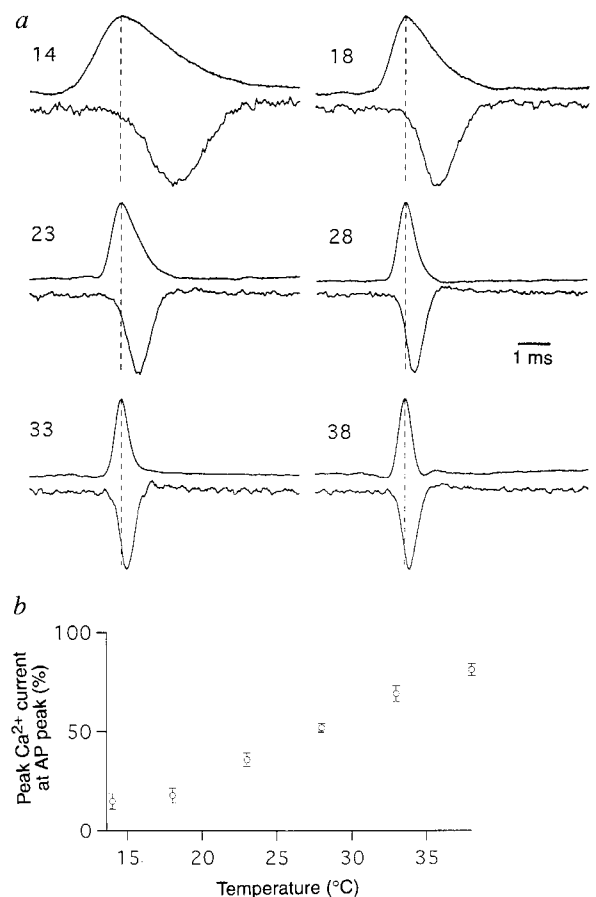


FIG. 3 Temperature dependence of presynaptic waveform and calcium current. a, Representative experiments showing the presynaptic waveform and calcium current at the indicated temperatures ($^{\circ}$ C). In each panel the top trace shows the presynaptic voltage waveform and the bottom the time course of the calcium current. The dashed vertical line is aligned with the peak of the action potential. b, Relative calcium influx at the peak of the action potential as a function of temperature (in order of increasing temperature $n = 5, 8, 13, 5, 4, 9$). The range of peak signal sizes (% $\Delta T/T$) was 0.1–0.8, 0.2–0.8, and 0.1–0.3 at 14 $^{\circ}$ C, 24 $^{\circ}$ C and 38 $^{\circ}$ C, respectively, and for (% $\Delta F/F$ ms $^{-1}$) was 4–7, 6–12, and 1–3.

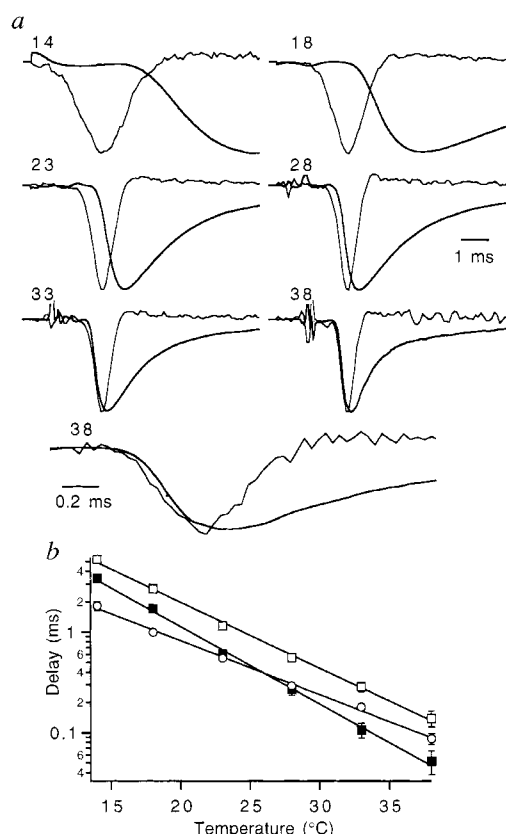


FIG. 4 Temperature dependence of synaptic delays. *a*, Presynaptic calcium current (first downward trace) and postsynaptic currents (second downward trace) recorded simultaneously at the indicated temperatures ($^{\circ}\text{C}$). Each trace is the average of 5 experiments, except at 14°C where $n = 3$. *b*, The delays from the start of the action potential to the start of calcium current (open circles), from the start of the calcium current to the start of the EPSC (solid squares) and the total delay from action potential to EPSC (open squares) are plotted as a function of temperature on a semilog plot. In many cases the error bars ($\pm$ s.e.m.) are smaller than the symbols. The corresponding lines are fits of the form $\ln(\text{delay}) = a - T/(\ln(Q_{10})/10)$ with a , Q_{10} : 2.27, 3.4; 3.67, 5.9; 3.70, 4.5).

Methods

Transverse slices were cut from the cerebellar vermis of 16–21-day-old rats and parallel fibres were focally loaded with calcium indicator as described previously^{5,6,21}. Stimulus-evoked fluorescence signals come almost entirely from presynaptic boutons, which account for an estimated 90% of the parallel fibre volume¹⁴. Parallel fibres were stimulated with a current pulse of 20–200 μs delivered through a glass pipette. All derivatives of magnesium green fluorescence have been inverted for clarity. The derivative of the fluorescence showed a small overshoot, which was due to a rapid component of decay of free calcium. An estimate of how fast an indicator responds to changes in calcium is given by the equilibration time constant of an indicator to a calcium step, $\tau = (k^+ + k^-)/([Ca] + [Ind])$ (ref. 16). The forward rate constant, k^+ , of these dyes is diffusion limited, therefore the differences in dye kinetics will be determined principally by the concentration of each dye, $[Ind]$, and the off rate, k^- . Because the dissociation constant $K_d = k^-/k^+$, lower-affinity dyes will tend to have faster kinetics. The reported affinities of the dyes used in this paper are: 200 nM for fura-2, 6 μM for magnesium-green, and 25 μM for mag-fura-5. Although high-affinity indicators, such as fura-2, can also be used to measure calcium currents¹⁵, this requires the introduction of larger concentrations of the dye than could be reliably achieved in our preparation. A direct comparison of the time course of the derivatives of magnesium green and mag-fura-5 fluorescence transients is not practical at 38°C because of the small signal size and the poor signal-to-noise ratio of mag-fura-5 signals. However, at higher temperatures the kinetics of the calcium indicators will be significantly faster¹⁶ and magnesium green and mag-fura-5 should continue to report accurately the time course of calcium currents.

Whole-cell voltage-clamp recordings of synaptic currents were made from stellate cells located in the outer third of the molecular layer with techniques similar to those described for Purkinje cells^{6,21}. Glass pipettes (2.5–4.0 M Ω) were used and access resistances were less than 10 M Ω after compensation.

Stimulus artefact correction was accomplished by subtracting the transient recorded at a holding potential of 0 mV. Sometimes a residual artefact remained that was well separated and easily distinguished from the synaptic current (see Fig. 2). In Fig. 2 the total concentration of divalent ions was kept constant by compensatory changes in the magnesium concentration.

Throughout this work the presynaptic waveform was measured with the voltage-sensitive dye RH482. This absorbance dye has been used previously to record presynaptic action potentials in cerebellar slices²⁸, and its optical properties allow recording of voltage (using 710 nm) while simultaneously measuring calcium transients with magnesium green (500DF20 excitation filter, 490/700BDR Dichroic, and 10SWF-650 (Newport) and LP530 emission filters). RH482 was loaded by bath application for 0.5 to 1 h at a concentration of 50–100 $\mu\text{g ml}^{-1}$. Similar results were obtained with other voltage-sensitive dyes (di-4-ANEPPS, di-8-ANEPPS, di-8-ANEPPQ, di-18:2-ANEPPS). The timing of the signal agreed well with the presynaptic volley measured electrically, in that the double derivative of the fluorescence signal coincided with the field recording. In most experiments voltage signals were measured in the presence of CNQX and AP5 to eliminate postsynaptic contributions. Even when postsynaptic responses were not blocked, as in Fig. 2, there was very little contamination from postsynaptic structures. A region centred over the stellate cell body was illuminated and transmittance and fluorescence were simultaneously monitored with two photodiodes. We used a 20- μm diameter spot that, based on comparison to signals recorded using different spot sizes, provided good signal-to-noise ratios without affecting signal timing. The signals from the photodiodes and the postsynaptic currents were processed in parallel and filtered identically (2 kHz filtering and 33 kHz sampling). Derivatives were calculated digitally with a second-order difference method that introduced no time delay. Voltage-sensitive dyes, such as those used in this study, have been shown to respond to changes in potential in less than 1.2 μs (ref. 29). Calcium indicators and voltage-sensitive dyes were obtained from Molecular Probes, with the exception of RH482 which was obtained from L. M. Loew.

With all voltage-sensitive dyes tested under all conditions, the fast component corresponding to the presynaptic action potential is followed by a sustained plateau that lasts for several hundred milliseconds. This component is unaffected by cadmium, CNQX or AP5 and is probably the result of potassium accumulation in the extracellular space³⁰ (B.L.S. and W.G.R., unpublished results) which depolarizes stimulated and unstimulated fibres. Because of its slow time course this component did not interfere with our ability to measure the presynaptic waveform. At low temperatures and for the second of two stimuli the magnitude of this sustained component was negligible. For aesthetic reasons, the 23, 28, 33 and 38°C panels show the action potential and calcium influx elicited by the second of two stimuli separated by 10 or 30 ms. The relative timing of the action potential and calcium current produced by the first and second stimuli was the same.

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Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis

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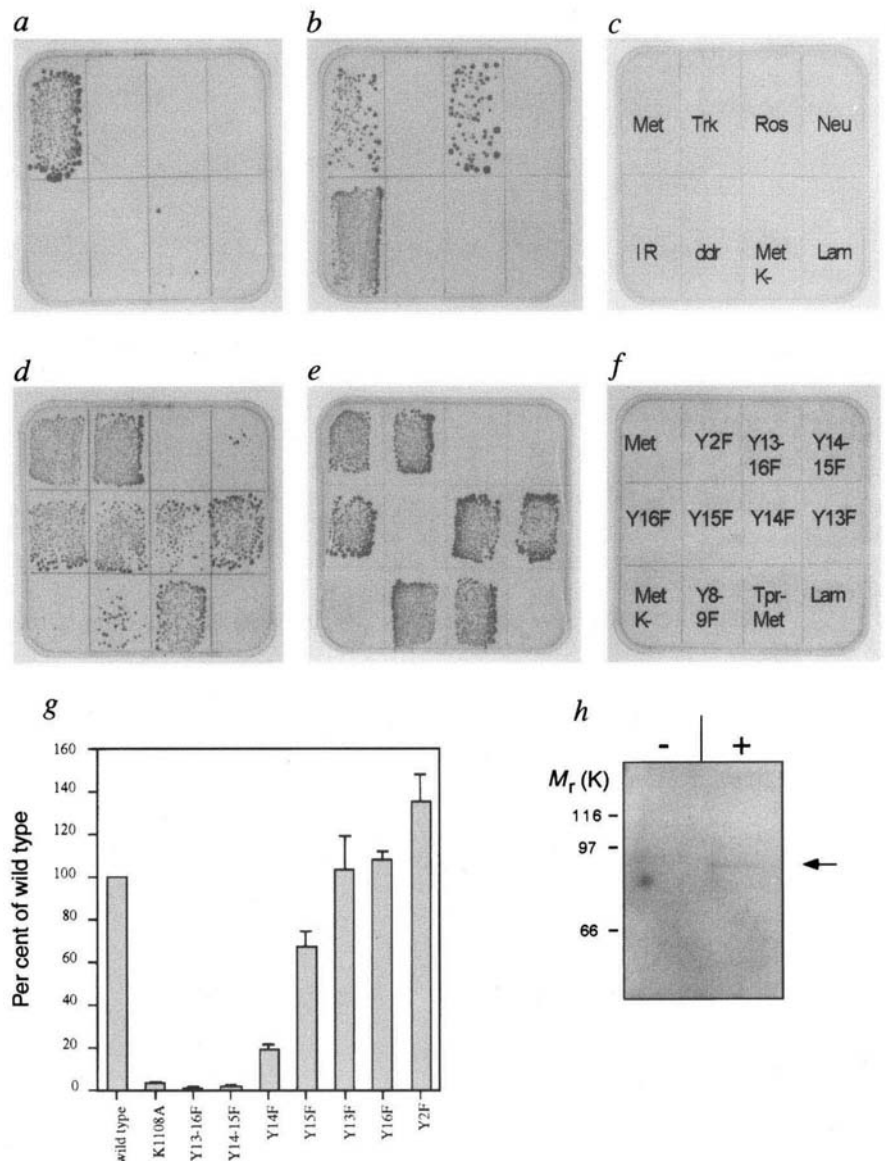
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THE proteins Gab1 and the related DOS (for 'daughter of sevenless') each bind to substrates of tyrosine kinases like Grb2 or Corkscrew, and act in signalling pathways downstream of tyrosine kinase receptors¹⁻³. Here we show that Gab1 interacts directly with the c-met-encoded receptor tyrosine kinase but not with a number of other tyrosine kinases from different subfamilies. A newly identified proline-rich domain of Gab1 is respon-

sible for the binding of this protein to the tyrosine-phosphorylated bidentate docking site^{4,5} in c-Met. Expression of Gab1 in epithelial cells is sufficient to induce the c-Met-specific activities⁶⁻⁹, including branching morphogenesis. Thus we have discovered a new phosphotyrosine interaction domain in Gab1 and shown that Gab1 is the substrate of the c-Met receptor tyrosine kinase that mediates epithelial morphogenesis.

We used a yeast two-hybrid screen¹⁰ to find specific substrates of the c-Met receptor tyrosine kinase. The hybrid bait consisted of the cytoplasmic part of c-Met and the DNA-binding and dimerization domain of LexA, and resulted in interchain phosphorylation of the receptor on tyrosine residues in yeast. In this way, we identified several complementary DNA clones, one of which encodes amino acids 391–532 of mouse Gab1. Human Gab1 has been isolated as a Grb2-binding protein¹ and has structural similarity to DOS and the insulin-receptor substrates (IRS)^{2,3,11}. We found that Gab1 interacts strongly with c-Met but not with other tyrosine kinases¹², for example TrkA, c-Ros, c-Neu, the insulin receptor and DDR (Fig. 1a; see Fig. 1c for the kinases tested), or with c-Ret, Sek-1, c-Kit, c-Abl, v-Sea, or the receptors for colony-stimulating factor-1, epidermal growth factor and keratinocyte growth factor (data not shown), demonstrating the specificity of the interaction. In contrast, other c-Met partners found in the screen (Grb2, Grb10, c-Abl, p85 and p55 forms of phosphatidylinositol-3-OH kinase (PI(3)K)¹²) bind to various tyrosine kinases (Fig. 1b, and data not

FIG. 1 Interaction of Gab1 with c-Met in the yeast two-hybrid system. **a–f**, Colony growth assay. **a**, Gab1 specifically interacts with c-Met (top left), but not with other tyrosine kinases (plan in **c** shows the tyrosine kinases tested; Met K⁻ is a kinase-inactive mutant; Lam is lamin, used as a control). **b**, The control p85 PI(3)K, interacts with c-Met, c-Ros and the insulin receptor (IR). **d**, Interaction of Gab1 with c-Met is dependent on the kinase being active and on the C-terminal tyrosine residues 14 and 15 (see **f** for the mutants tested⁵ and **g** for quantitative data). **e**, Binding of Grb2 to c-Met requires only tyrosine residue 15 (mutants tested shown in **f**). **g**, Quantification of the interaction of Gab1 with c-Met and mutants using a β -galactosidase assay. The cytoplasmic tyrosine residues of c-Met (numbered from 1 to 16, starting from the juxtamembrane region) were mutated to phenylalanine (F) as follows⁵: Y2F (the second Y of the receptor at amino-acid position 1,001), Y13–16F (positions 1,311, 1,347, 1,354 and 1,363), Y14–15F (positions 1,347 and 1,354), Y16F (position 1,363), Y15F (position 1,354), Y14F (position 1,347), Y13F (position 1,311) and Y8–9F (positions 1,232 and 1,233). In Met K⁻, lysine at residue 1,108 was mutated to alanine (K1108A). β -Galactosidase activity from five independent transformations was measured in Miller units²⁵. **h**, Far-western blot demonstrating the direct interaction of Gab1 with c-Met (arrow). The blot shows extracts from control (–), LexA-lamin and LexA-Met transfected yeast cells (+) probed with a purified GST–Gab1 (amino acids 391–532) fusion protein and developed using anti-glutathione-S-transferase (GST) antibodies and iodinated protein A.



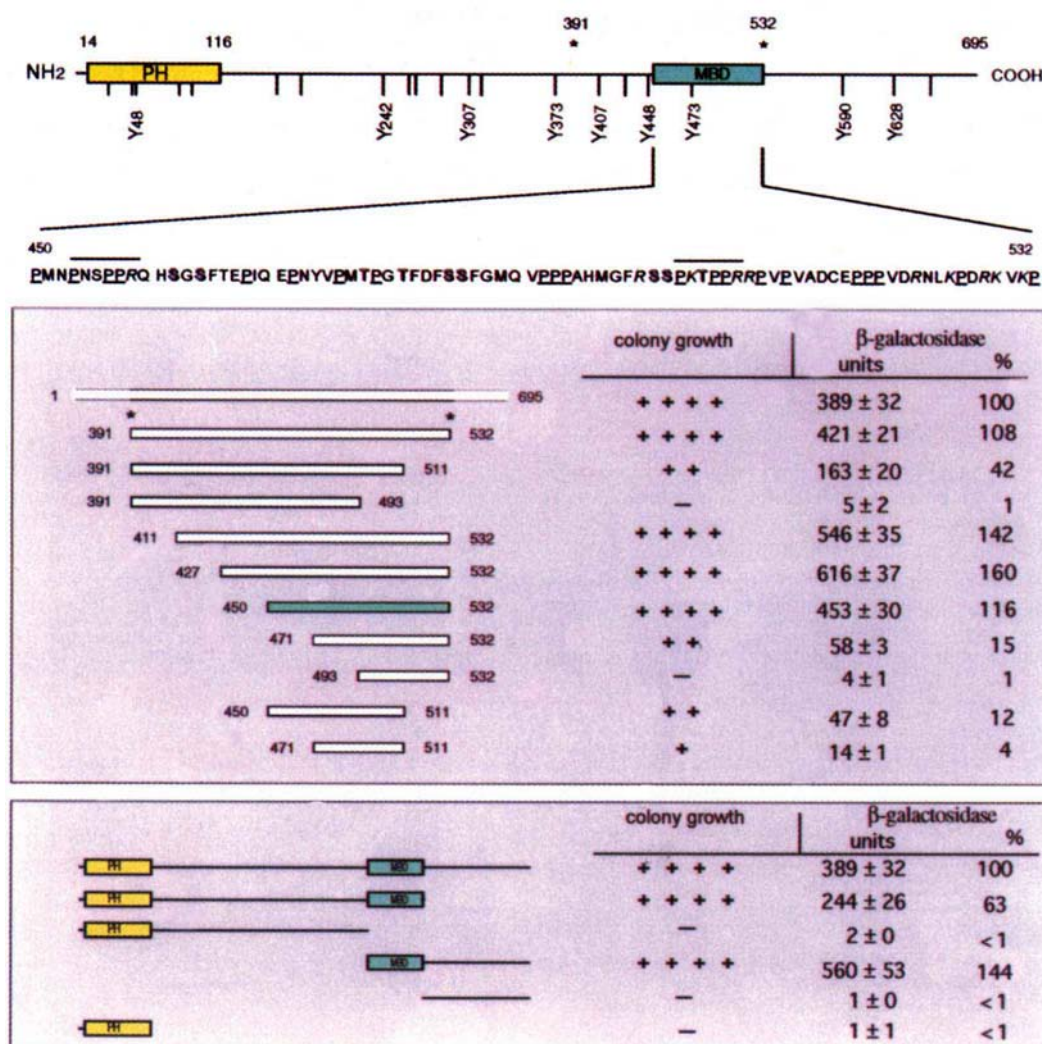


FIG. 2 The domain of Gab1 that mediates association with c-Met in the yeast two-hybrid system. Full-length Gab1 and its deletion mutants are shown: progressive deletion of the N- and C-terminal regions defined the 83-amino-acid binding domain of Gab1 (highlighted as MBD, for c-Met-binding domain). Assays were done as for Fig. 1. Diameter of yeast colonies: + + + +, >1 mm in 2 d; + +, >1 mm in 4 d; +, >1 mm in 6 d; -, <1 mm in 8 d. PH, pleckstrin-homology domain of Gab1. Tyrosine

residues with defined consensus sequences for binding sites of SH2 domains are indicated⁴; other tyrosine residues are marked by vertical bars. Asterisks mark the Gab1 sequence identified in the initial two-hybrid screen. The amino-acid sequence of the MBD is shown in single-letter code; proline (underlined), serine and threonine (shell letters) residues are indicated. Consensus sequences for binding sites of SH3 domains of Grb2 are marked above the sequence by horizontal bars.

shown). Signal transduction by c-Met depends on its kinase activity and the phosphorylation of two closely spaced tyrosine (Y) residues at positions 14 and 15 in the carboxy-terminal region (Y14, amino-acid position 1,347 and Y15, position 1,354)^{4,5,13}. Gab1 did not interact with a mutant c-Met lacking kinase activity, binding was impaired when Y14 or Y15 were mutated, and mutation of both tyrosine residues prevented binding (Fig. 1d; compare with *f* for the tested c-Met mutants, and with *e* for interaction of Grb2 with c-Met). Tpr-Met, an oncogenic variant of c-Met¹⁴, also bound Gab1 (Fig. 1d). Direct interaction of Gab1 with c-Met was detected in a far-western blot using a GST-Gab1 fusion protein as probe (Fig. 1h). The domain of Gab1 necessary and sufficient for binding of Gab1 to c-Met (Fig. 2) was an 83-amino-acid sequence (residues 450–532; highlighted as MBD, for c-Met-binding domain). The MBD of Gab1 is rich in proline (25%), serine/threonine and arginine/lysine residues. These results demonstrate the suitability of our yeast two-hybrid system for identifying new substrates of tyrosine kinases and for mapping specific interaction sites.

The interaction of c-Met with Gab1 was also investigated in mammalian cells by reciprocal co-immunoprecipitation. After

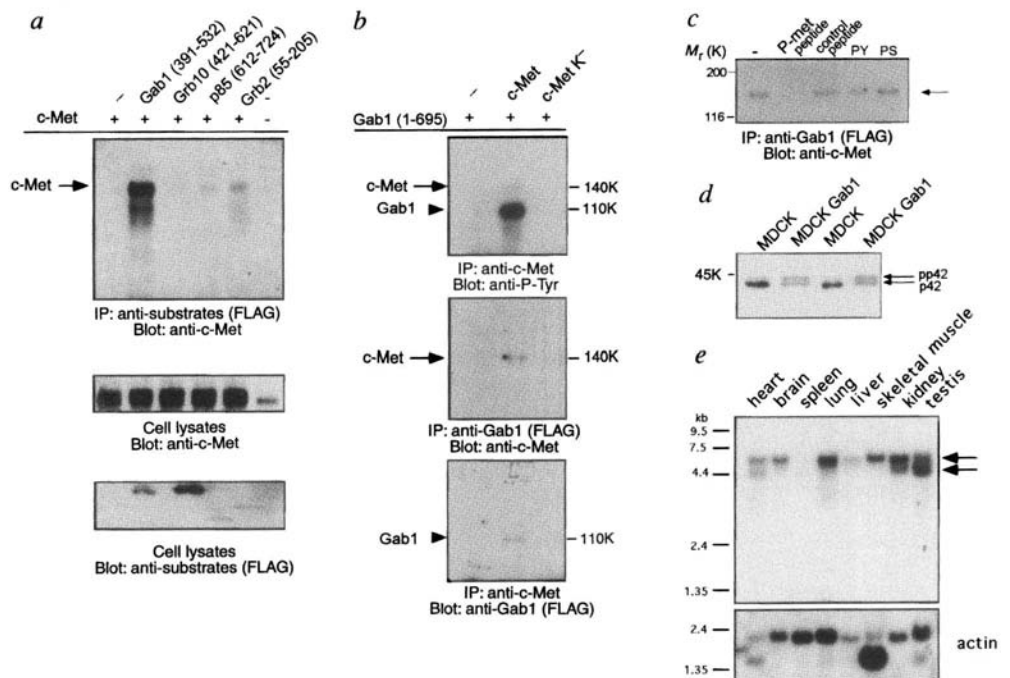
activation, large amounts of c-Met were immunoprecipitated with the c-Met-binding domain of Gab1 (Fig. 3a); the binding of c-Met to the SH2 domains of Grb2, p85 PI(3)K and Grb10 was weaker. Full-length Gab1 also co-immunoprecipitated with c-Met and its tyrosine residues were phosphorylated (Fig. 3b). Interaction of Gab1 with c-Met was specifically blocked by a synthetic 24-amino-acid phosphopeptide of c-Met sequence containing tyrosine residues 14 and 15 (Fig. 3c). Northern blotting revealed high expression of Gab1 in lung, kidney, testis, liver, brain, heart and skeletal muscle of the mouse (Fig. 3e; see also ref. 1), organs that express *c-met*¹⁵. There are two messenger RNA species of Gab1 which are differentially expressed.

We next determined the role of Gab1 in the transduction of biological signals from the c-Met receptor tyrosine kinase. In tissue culture, c-Met activated by its ligand scatter factor/hepatocyte growth factor (SF/HGF) induces dissociation and increased motility of epithelial cells^{6,7}. When such cells are cultured in three-dimensional collagen matrix in the presence of SF/HGF, branching tubules are formed, a process resembling epithelial morphogenesis during development^{7,9,16–19}. We found that overexpression of full-length Gab1 in kidney epithelial cells, which is strongly

phosphorylated on tyrosine residues, results in constitutive and ligand-independent cell scattering and branching morphogenesis (Fig. 4a, c, e). Expression of Gab1 resulted in activation of MAP kinase (Fig. 3d). Confocal microscopy showed that Gab1 is preferentially located at cell-to-cell contact sites (arrows), whereas distribution of an actin control is circumferential (Fig.

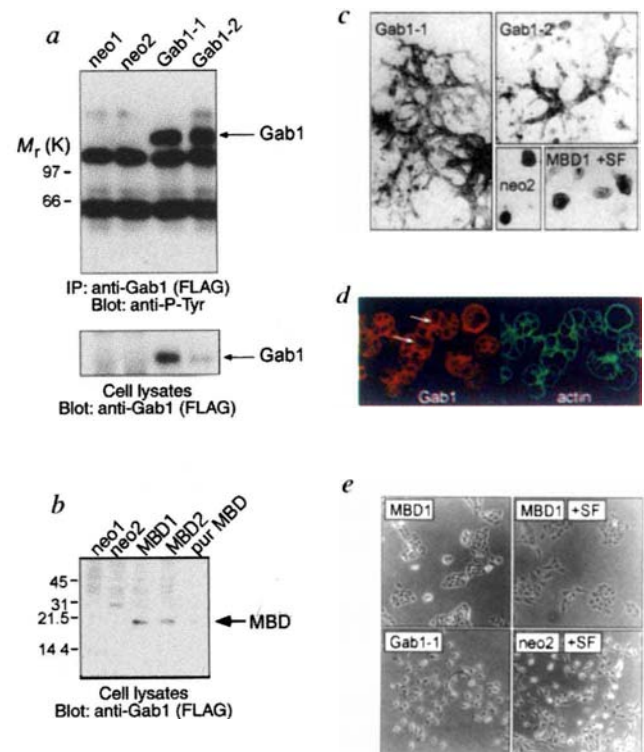
FIG. 3 Interaction and phosphorylation of Gab1 by c-Met in mammalian cells and tissue distribution of Gab1. **a**, Co-immunoprecipitation of c-Met with the binding domains of Gab1, Grb10, p85 PI(3)K and Grb2 (upper gel). Binding of Gab1, Grb2 and p85 to c-Met was 100, 7 and 4%, respectively; optical densities were scanned and integrated using the NIH-image program, version 1.59. Binding domains of substrates were identified in the yeast two-hybrid screen and were epitope-tagged (Flag; Kodak). Expression of c-Met and the substrates is shown in the lower gels. IP, immunoprecipitate. **b**, Association of full-length Gab1 depends on active c-Met kinase. The co-immunoprecipitated Gab1 (arrowhead) is strongly phosphorylated on tyrosine residues (top gel; compare with phosphorylated c-Met, arrow); minus sign, no receptor added; c-Met K⁻, kinase-inactive mutant of c-Met. Lower gels show reciprocal co-immunoprecipitation of c-Met and Gab1. **c**, Competition for binding of Gab1 to c-Met by a tyrosyl phosphopeptide of c-Met (IFSTFGE-HY*VHVNATY*VNVKCA, where Y* indicates phosphotyrosine). Peptides (50 µM) and controls (phosphotyrosine (PY) and phosphoserine (PS), 10 mM) were added before co-immunoprecipitation. **d**, MAP kinase activity in two control and Gab1-transfected MDCK cell clones was analysed using anti-panERK antibodies (Transduction Labs). MAP kinase activity is increased in Gab1 transfectants,

4d). In contrast, overexpression of truncated Gab1 containing only the c-Met-binding domain strongly impaired the response to c-Met signals (Fig. 4b, c, e). These results indicate that activation of Gab1 alone is sufficient for the induction of the pleiotrophic cellular responses that are characteristically seen after c-Met activation.



as indicated by the partial upshift of the 42K band (p42 → pp42). **e**, Northern blot containing poly(A)⁺ RNA from various mouse tissues was hybridized with Gab1 cDNA. Molecular size markers are shown on the left. Arrows indicate the two transcripts of Gab1.

FIG. 4 Gab1 mediates the biological signals of c-Met in epithelial cells. **a**, Western blot showing the expression and phosphorylation of full-length Gab1 in stably transfected MDCK cells (clones Gab1-1 and Gab1-2). Arrows indicate Gab1 with a relative molecular mass of 110,000. Neo transfectants were used as controls (clones neo1 and neo2). For immunoprecipitation and western blotting, antibodies indicated were used (see also Fig. 3). **b**, Western blot showing the expression of the c-Met-binding domain of Gab1 (amino acids 391–532) in stably transfected MDCK cells (clones MBD1 and MBD2). Arrow marks the c-Met-binding domain of Gab1; purified recombinant MBD (pur MBD) was used as a marker. Neo transfectants were used as controls (neo1 and 2). **c**, Gab1 induces constitutive, ligand-independent branching morphogenesis in collagen matrices. Tubule formation is seen in transfectants overexpressing Gab1 (clones Gab1-1 and Gab1-2); control transfectants (neo2) form only smooth cysts as parental cells. Moreover, SF/HGF-induced branching morphogenesis is inhibited in MDCK cells expressing the MBD (MBD1). **d**, Confocal immunofluorescence microscopy showing full-length Gab1 at cell-to-cell contact sites. Cortical actin is located at all cell surfaces. Staining was with anti-Flag antibodies for Gab1 and FITC-phalloidin for actin. **e**, The c-Met-binding domain of Gab1 inhibits SF/HGF-induced scattering of MDCK epithelial cell colonies in a dominant-negative manner (mobility shown for clone MBD1). Neo transfectants are readily dissociated by SF/HGF (neo2). Moreover, constitutive cell scattering is seen in MDCK cells overexpressing Gab1 (shown for clone Gab1-1).



Gab1 is a member of a family of multiadaptor proteins that includes DOS and IRS-1 and IRS-2 (insulin-receptor substrates)^{1-3,11}. This family is characterized by a pleckstrin-homology domain²⁰ at the amino terminus and multiple binding sites for SH2 and SH3 domains. We have identified a new phosphotyrosine-interaction domain in Gab1 (MBD, from amino acids 450–532) which binds with an as-yet unique specificity to a c-Met tyrosyl phosphopeptide. In addition to SH2 and PTB domains²¹⁻²³, the MBD domain of Gab1 is another example of a protein module that recognizes phosphotyrosine-containing peptides. Our findings support the idea that the specificity of signalling by different tyrosine kinase receptors depends on interaction with specific substrates as well as with substrates shared by several receptors. □

Methods

Yeast two-hybrid screens. cDNAs encoding the cytoplasmic regions of c-Met (nucleotides 2,865 to 4,137; ref. 7) and other receptor tyrosine kinases¹² were fused to LexA sequences in the yeast expression vector BTM116. The hybrid cDNAs were transfected into yeast strain L40 (which contains a *HIS3* and *lacZ* reporter gene) and assayed for interaction with proteins encoded by a VP16-activation-domain cDNA library from E10.5 mouse embryos²⁴. Other partners of c-Met found in the yeast two-hybrid screen besides Gab1 were Grb2 (amino acids 55–205), p85 PI(3)K (amino acids 612–724), p55 PI(3)K (amino acids 1–461), Grb 10 (amino acids 421–621) and c-Abl (amino acids 123–223)¹². Full-length Gab1 isolated from a λ gt11 mouse embryo cDNA library (ML 1027a; Clontech) had an open reading frame of 695 amino acids and shared 90% amino-acid identity with human Gab1 (ref. 1). Deletion mutants of Gab1 were made by polymerase chain reaction (PCR) using specific primers at convenient intervals. Gab1 fragments were introduced into the *Not*I restriction site of the yeast expression plasmid pVP16 (ref. 24).

Characterization of mouse Gab1. cDNAs of Gab1 and other c-Met-binding proteins were inserted into pBAT Flag²⁴ and cotransfected with *trk-met*⁷ into neuro2A cells; Trk-Met protein mediates all c-Met-specific signals in response to nerve growth factor (NGF)⁷. After two days, transfectants were stimulated with NGF for 5 min to activate Trk-Met and then lysed in 1% Triton X-100 containing protease inhibitors and phosphatase inhibitors. For immunoprecipitation and western blotting, anti-c-Met (Santa Cruz), anti-Flag (Kodak) and anti-phosphotyrosine (PY20; ICN Biomedicals) antibodies were used. Proteins were visualized with peroxidase-conjugated secondary antibodies (Dianova) and enhanced chemiluminescence (Amersham). The multiple-tissue northern blot (Clontech) was probed with the ³²P-labelled Gab1 cDNA encoding amino acids 391–532. The cDNAs of full-length Gab1 and the c-Met-binding domain in pBAT Flag were stably expressed in MDCK cells together with a neomycin-resistance gene (pSV2neo). Cell motility (scattering) and branching morphogenesis were assayed as described¹⁹; SF/HGF was added at 20 ng ml⁻¹.

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Biochemical evidence that Patched is the Hedgehog receptor

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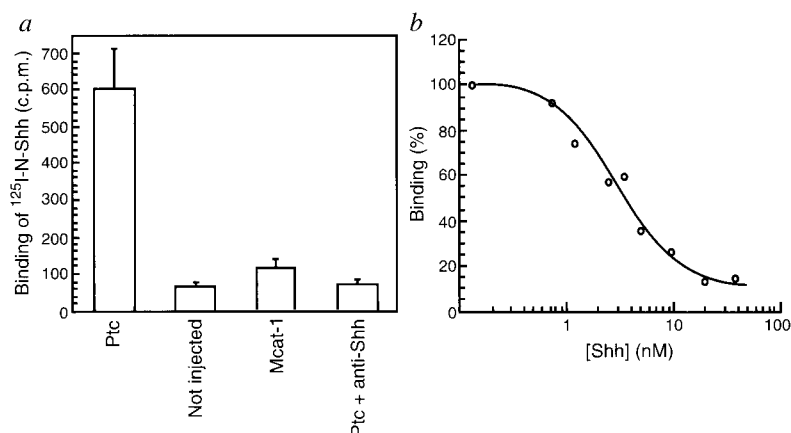
THE protein Sonic hedgehog (Shh) is essential for a variety of patterning events during development. It is the signal from the notochord that induces ventral cell fate in the neural tube and somites^{1,2}, and is the polarizing signal for patterning of the anterior–posterior axis of the developing limb bud³. Because of these and other inductive functions of Shh, it is important to understand how the Hedgehog (Hh) signal is received by the target cells. Here we describe binding studies using labelled Shh that strongly suggest that the Hh receptor is encoded by *patched* (*ptc*), a gene first identified in genetic screens in *Drosophila*⁴.

Epistasis analysis has implicated a number of genes as acting downstream of *hh* in *Drosophila*⁵, including *patched* (*ptc*)^{6,7}. Transcription of *hh* targets is repressed in cells expressing *ptc*, and *hh* acts to antagonize *ptc* activity, resulting in the induction of the target genes^{8–10}. Although it acts downstream of *hh*, *ptc* seems to act upstream of all other genes known in the pathway, including *smoothed* (*smo*)^{11,12}, *fused* (*fu*) and *cubitus interruptus* (*ci*)³. Moreover, sequence analysis and antibody studies indicate that the protein Ptc lies in the cell membrane^{13,14}, suggesting that Ptc functions as the Hh receptor⁹. However, the putative protein structure is not reminiscent of any known class of receptors.

The vertebrate homologue of *ptc* has recently been cloned from chick¹⁵, mouse¹⁶ and human¹⁷, and seems to be expressed at sites of vertebrate Hh signalling^{15–16}. To address the possibility of Ptc functioning as the Shh receptor, we tried to compare binding of labelled Shh to non-expressing and *ptc*-transfected cells. Unfortunately, several of the mammalian cell lines tested, including NIH3T3 and C3H10T1/2 cells, constitutively express *Ptc*. However, data from mouse suggest that germline cells do not express *Ptc*¹⁸. We therefore expressed chick *Ptc* in *Xenopus laevis* oocytes. Protein of the expected size was detected in the oocyte extract by western blot 48 h after injection of *ptc* messenger RNA (data not shown). We performed binding assays on injected, uninjected and control-injected oocytes using human recombinant amino-terminal fragment of Shh protein (N-Shh) produced in *Escherichia coli* and labelled with ¹²⁵I. The binding assay showed that the labelled N-Shh protein could bind oocytes injected with *ptc* mRNA but not oocytes injected with an mRNA encoding a control cell-surface protein (Mcat-1)¹⁹ or uninjected oocytes (Fig. 1a). Binding to oocytes injected with *ptc* mRNA was observed with two different labelled Shh proteins: human N-Shh produced in *E. coli*, and mouse N-Shh produced using the baculovirus system (data not shown).

We tested whether binding of iodinated N-Shh to oocytes injected with *ptc* mRNA is specific both by competing the binding of labelled N-Shh with unlabelled N-Shh, and by using a blocking antibody specific for N-Shh. We found that unlabelled N-Shh could compete specifically the binding of labelled N-Shh (Fig. 1b), but the binding could not be competed by a control protein, β -galactosidase (data not shown). We also found that baculovirus-produced N-Shh could compete with the binding of labelled bacterially produced protein (data not shown), indicating that the radioactive protein binding to oocytes injected with *ptc* mRNA is indeed N-Shh and not a contaminant in the bacterial protein preparation.

FIG. 1 a, Binding of ^{125}I -N-Shh to oocytes injected with *ptc* mRNA is compared to binding to uninjected oocytes and oocytes injected with *mcad-1* mRNA encoding a control protein localized in the cell surface. Binding of labelled N-Shh to oocytes injected with *ptc* mRNA is significantly inhibited ($P < 0.001$) in the presence of a monoclonal antibody recognizing N-Shh (anti-Shh). b, Competition of ^{125}I labelled N-Shh (2.5 nM) binding adding increasing amounts of unlabelled N-Shh. Binding is reported as percentage of binding, considering 100% to be the level of binding in the absence of competitor.



To determine whether the observed N-Shh-Ptc interaction is biologically relevant, we investigated whether the domain of N-Shh involved in the binding to Ptc is the same domain that is responsible for N-Shh biological activity. Binding could be inhibited by adding a blocking monoclonal antibody to the binding reaction at the same concentration that blocks Shh biological activity²⁰ (Fig. 1a). However, binding of labelled N-Shh was not inhibited by addition of polyclonal antibody which recognizes Shh in immunohistochemistry but does not block biological activity (data not shown). Because the blocking antibody interferes with the transduction of the Shh signal *in vivo* as it interferes with the binding to Ptc, the same epitope of the protein is involved in both processes. This strongly suggests that Ptc is a functional receptor for Shh in its signalling.

To define conditions to study the binding reaction at equilibrium, we performed time-course experiments with excess N-Shh. A plateau was reached after 30-min incubation at room temperature, similar to the plateau reached at 4 °C in parallel experiments (data not shown). We therefore used a 35-min incubation at room temperature in all the following experiments to work at equilibrium binding conditions.

By adding increasing amounts of labelled N-Shh we demonstrated that binding of N-Shh to oocytes injected with *ptc* mRNA was saturable, reaching saturation at a concentration of 10 nM N-Shh (Fig. 2a, b). Scatchard analysis of this data (Fig. 2c) indicates that the dissociation constant (K_d) for the interaction between Ptc and N-Shh is $\sim 1.2 \times 10^{-9}$ M. This is the same order of magnitude of the concentration of N-Shh required for biological effect *in vivo*²⁰. The analysis also indicates that approximately 3×10^9 receptors are present per oocyte injected with *ptc* mRNA. This is similar to the reported number of EGF receptors synthesized per oocyte in a similar experiment²¹. This high number of receptors supports the idea that Ptc itself binds N-Shh directly, rather than activating an endogenous *Xenopus* receptor protein which would be present at much lower levels. However, we cannot exclude the possibility that Ptc associates with other proteins that affect Shh binding affinity.

The prediction of a direct interaction between Ptc and Shh was confirmed by using co-immunoprecipitation. We found that the binding characteristics of an epitope-tagged variant of Ptc were identical to those of wild-type Ptc in the oocyte system (data not shown). Oocytes were therefore injected with mRNA encoding Ptc in frame with an influenza haemagglutinin (HA)-epitope tag at the carboxy terminal and incubated with ^{125}I -N-Shh. Subsequently, the oocyte extract was immunoprecipitated with an anti-HA antibody. Labelled N-Shh could be detected just in extracts from oocytes injected with mRNA for Ptc and not from uninjected oocytes, nor from oocytes injected with control mRNA encoding a different transmembrane protein (Mcat-1) bearing the same HA-epitope tag (Fig. 3).

After demonstrating specific binding of N-Shh to Ptc, we

wanted to define which domains of Ptc are required for the interaction. Ptc has been predicted to span the plasma membrane 12 times, with a symmetrical conformation of six-plus-six transmembrane domains^{7,16}. The symmetry is revealed by two big extracellular loops. If the predicted protein conformation is correct, one or both of the extracellular loops are likely to be involved in the binding to N-Shh. We investigated whether the two putative extracellular loops were required for N-Shh binding by analysing three mutations of the Ptc. We deleted either the first (PTCΔLoop1), the second (PTCΔLoop2), or both of the two extracellular loops (PTCΔLoop1 + 2) (Fig. 4a). After injection of mRNAs for the three different constructs, we analysed the proteins translated by the oocytes and confirmed that the

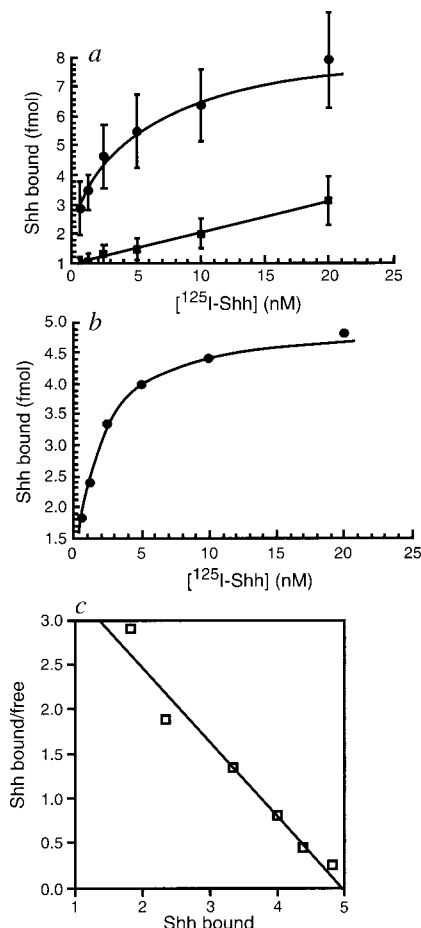
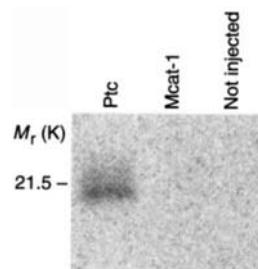


FIG. 2 a, Oocytes injected with *ptc* mRNA (circles) or control uninjected oocytes (squares) were treated with increasing amounts of labelled N-Shh. Each point represents the mean binding to 20 individual oocytes from four independent experiments. Adding increasing amounts of labelled N-Shh led to some additional, apparently nonspecific, binding. b, Specific binding of ^{125}I labelled N-Shh to oocytes expressing Ptc. This curve is derived from data in a by subtraction of the non-specific binding curve measured in uninjected oocytes from Ptc-expressing oocyte curve. The binding reaches saturation at an N-Shh concentration of 10 nM. c, Data from b presented as a Scatchard plot. The line shown was generated by linear regression. The K_d is approximately 1.2×10^{-9} M, and B_{max} is approximately 3×10^9 sites per oocyte injected with *ptc* mRNA.

FIG. 3 125 I-labelled N-Shh co-immunoprecipitates with Ptc protein. Oocytes expressing a tagged form of Ptc were incubated with 125 I-labelled N-Shh. The cell extract was immunoprecipitated with an anti-HA tag. The immunoprecipitated proteins were separated in a denaturing polyacrylamide gel (15%) under reducing conditions. 125 I-labelled N-Shh could be detected in extracts from oocytes injected with *ptc* mRNA but not from oocytes injected with mRNA encoding a control tagged protein (Mcat-1), nor from uninjected oocytes.



mutant proteins were stable and processed with the expected molecular masses (Fig. 4b). The mutated proteins were also glycosylated, as shown by a change in electrophoretic mobility following tunicamycin treatment (data not shown), demonstrating that they are processed through the secretory pathway in the oocytes. We then performed binding assays on oocytes expressing the three mutated forms of Ptc and found that the binding was significantly affected. The binding of labelled N-Shh to the three mutants was not statistically different from the control uninjected oocytes (Fig. 4c). These data indicate that the predicted Ptc topology is likely to be correct, and that the two extracellular loops are both required for the binding of N-Shh to Ptc, either directly in contacting the ligand, or for the proper conformation of the binding domain of Ptc.

Comparison of the Ptc protein sequence from different species showed that two predicted glycosylation sites in the two extra-

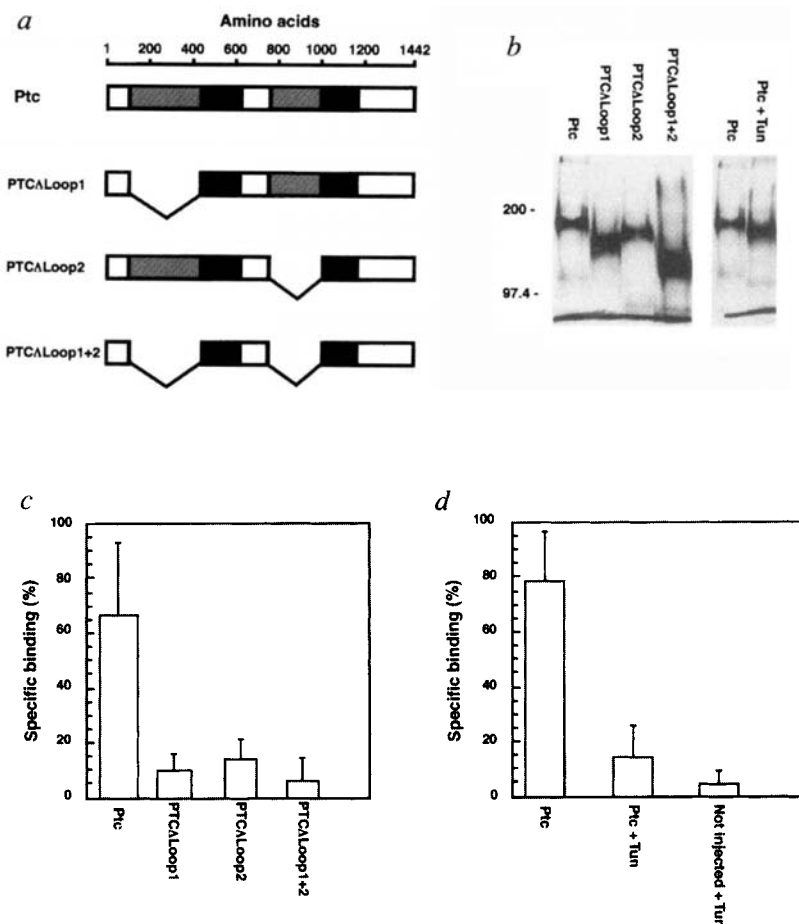
cellular loops are highly conserved in evolution¹⁶. To investigate whether glycosylation of Ptc is required for N-Shh binding, we inhibited Ptc glycosylation by treating the injected oocytes with tunicamycin. We analysed the size of Ptc by western blot after tunicamycin treatment, and detected a shift to a lower molecular weight as expected for the non-glycosylated protein (Fig. 4b). Binding of N-Shh decreased significantly ($P < 0.001$) when glycosylation of Ptc was inhibited (Fig. 4d). The binding of labelled N-Shh to oocytes expressing Ptc and treated with tunicamycin was not significantly different from the nonspecific binding to uninjected oocytes, whether treated with tunicamycin or not (Fig. 4d).

The predicted structure of Ptc is reminiscent of the six-plus-six family of transporters²². This raises the possibility that Ptc could function as a ligand-gated channel. However, in preliminary studies we were unable to detect any Na^+ , Ca^{2+} , Cl^- or K^+ currents in the presence or absence of N-Shh (V. M. and K. Schwartz, data not shown).

The crystallographic structure of Shh suggests that the N-terminal fragment possesses protease activity, which could act in freeing the ligand from the surface of the cells in which it is synthesized, or in activating a target protein by cleavage²³. We therefore considered the possibility that, as well as binding to Ptc, N-Shh might also proteolytically activate it. We analysed the size of epitope-tagged Ptc after incubation with saturating amounts of N-Shh, but could not detect a shift in the migration of Ptc compared to the protein from oocytes not treated with N-Shh (data not shown).

Although the putative Shh binding extracellular loops and the predicted transmembrane domains are highly conserved between the vertebrate and fly proteins, the intracellular domains exhibit lower homology^{15,16}. This raises the possibility that, instead of signalling directly into the cell, Ptc might interact with another

FIG. 4 a, Schematic representation of Ptc protein and Ptc deletion constructs. Amino acids are numbered as shown. The predicted extracellular loops are shown in grey and the transmembrane domains in black. PTCΔLoop1 is a deletion from amino acid 119 to 438. PTCΔLoop2 is a deletion from amino acid 768 to 1026. In PTCΔLoop1 + 2, amino acids 119–438 and 768–1026 have been deleted. b, Western blot analysis of the Ptc proteins translated in oocytes injected with mRNA encoding wild-type Ptc, PTCΔLoop1, PTCΔLoop2 and PTCΔLoop1 + 2. The proteins are tagged with an HA-epitope tag. After separation in a 6% denaturing polyacrylamide gel and transfer to nitrocellulose, the proteins were detected using an anti-HA tag antibody. Right, a comparison of the size of Ptc extracted from oocytes expressing *ptc* mRNA treated (PTC + Tun) or not treated with tunicamycin. c, Histogram representing the percentage of specific binding (after subtraction of nonspecific binding measured in uninjected oocytes) of labelled N-Shh to oocytes expressing Ptc or with PTCΔLoop1, PTCΔLoop2, PTCΔLoop1 + 2. The binding to the mutated forms of Ptc is not significantly different from the background of uninjected oocytes. d, Glycosylation is required for N-Shh binding to Ptc. Binding of labelled N-Shh is represented as percentage of specific binding after subtraction of background measured with uninjected oocytes. When glycosylation is blocked by treatment with tunicamycin, binding to oocytes injected with *ptc* mRNA is not significantly different than the binding to uninjected oocytes treated or not treated with tunicamycin.



membrane-bound partner. A candidate for such a protein is that encoded by the recently cloned *Drosophila* gene *smoothed* (*smo*)^{12,24}. Smo is a seven-transmembrane protein acting downstream of *ptc* in the *hh* pathway. A model consistent with current genetic data would be that Ptc binds to Smo, inactivating it. Hh binding to Ptc could result in the release of Smo, allowing it to activate targets. The same target activation would result from the loss of Ptc, as has been observed^{8,9,10}. Further biochemical studies will be required to address this and other possibilities. The identification of Ptc as part of the Shh receptor complex will allow the enigmatic Hh signalling pathway to be studied in more detail, and its role in regulating embryonic patterning to be understood. □

Methods

Binding assay. The open reading frame of the chick *Ptc* gene was cloned into the pRD67 and pRD67:HA vectors (two derivatives of pSP64T vector containing an SP6 promoter and 5' and 3' untranslated sequences from *Xenopus laevis* β -globin). In pRD67:HA an *Xho*I site was used to clone three copies of the HA-tag epitope peptide in frame with the last amino acid of the *ptc* coding sequence. *In vitro* transcription of capped mRNA was performed using mMESSAGE mMACHINE (Ambion). mRNA (25 ng) was injected into freshly collected and defolliculated oocytes. The oocytes were washed 48 h after injection in 5 mM HEPES, pH 7.6, 5 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ (binding buffer) and blocked by adding 1% BSA in binding buffer for 1 h at room temperature. The binding assay on injected or uninjected oocytes was performed in 50 μ l 5% BSA in binding buffer at room temperature. Purified human Sonic hedgehog protein was supplied by Ontogeny (Cambridge). The bacterially produced protein used in most of the experiments was 99% pure and shown to be active in biological signalling by the supplier. Mammalian Shh protein is functionally equivalent to the chick gene product, causing pattern duplications in the chick wing²⁵ and inducing ventral cell fate in chick neural tube and somite explants^{20,26,27}. Furthermore, the N-terminal fragment of the human Shh protein, which is sufficient to mediate signalling^{20,25,28,29}, is 98.8% identical to the chick N-Shh protein. N-Shh was labelled with ¹²⁵I (Amersham) using Iodo-beads (Pierce). The unlabelled and labelled protein solutions were both quantified by colorimetric assay (BioRad). After 35-min incubation with labelled protein, the oocytes were washed twice with 1% BSA in binding buffer and three times with binding buffer on ice. Each oocyte was transferred to a vial and counted individually in a gamma counter.

Deletion constructs. Deletion of the first extracellular loop of chick *Ptc* protein from amino acids 119–438 (producing PTC Δ Loop1) was obtained by restriction digestion with *Bsp*HI and *Eco*NI, followed by Klenow polymerase filling of the protruding ends. The blunt ends were then ligated using T4 ligase. Deletions of the second extracellular loop from amino acids 768–1,026 of *Ptc* (producing PTC Δ Loop2) or of PTC Δ Loop1 (producing PTC Δ Loop1 + 2) were obtained by restriction digestion with *Bgl*II and *Bst*EII, followed by Klenow polymerase filling of the protruding ends and ligation with T4 ligase.

Tunicamycin treatment. Oocytes were incubated with tunicamycin (2 μ g ml⁻¹) for 24 h before injection. For injection, *ptc* mRNA was dissolved in water containing 4 μ g ml⁻¹ tunicamycin. Tunicamycin was maintained at the concentration of 2 μ g ml⁻¹ in the oocyte culture medium until the binding assay.

Co-immunoprecipitation. Crosslinking is problematic in the oocyte system because of the existence of the vitelline membrane surrounding the oocyte which interacts with the reactive group on the crosslinking reagent before it can reach the cell membrane. Nonetheless, we attempted to crosslink ¹²⁵I-N-Shh by sequential treatment with two reagents. After N-Shh binding, as described above, oocytes injected with mRNA encoding HA-tagged *Ptc*, or with mRNA encoding a control transmembrane protein (Mcat-1), also HA-tagged, or uninjected, were treated with 10 mM NHS-acetate (Pierce) in 10 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂ at 4 °C for 1 h. The oocytes were subsequently incubated with 1 mM DSP crosslinking reagent (Pierce) at 4 °C for 30 min. After stopping the reaction with binding buffer (which contains Tris), the oocytes were extracted with 10 mM CHAPS in binding buffer. The extract was treated with anti-HA antibody (12CA5, Boehringer Mannheim) and Protein A-Sepharose (Pharmacia) at 4 °C for 1 h and then washed with 10 mM CHAPS in binding buffer. After separation in a 15% denaturing polyacrylamide gel in the presence of β -mercaptoethanol, which reduces the disulphide bonds produced in crosslinking, ¹²⁵I-N-Shh migrated with an *M_r* of just less than 20K, the expected mobility of the denatured protein. However, the ability to immunoprecipitate N-Shh with epitope-tagged *Ptc* was independent of the presence of crosslinking reagents. This was verified by running the cross-linked extracts in a 5% polyacrylamide gel under non-reducing conditions, where no shift to a higher *M_r* was observed (data not shown).

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CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5

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For efficient entry into target cells, primary macrophage-tropic and laboratory-adapted human immunodeficiency viruses type 1 (HIV-1) require particular chemokine receptors, CCR-5 and CXCR-4, respectively, as well as the primary receptor CD4 (refs 1–6). Here we show that a complex of gp120, the exterior envelope glycoprotein, of macrophage-tropic primary HIV-1 and soluble CD4 interacts specifically with CCR-5 and inhibits the binding of the natural CCR-5 ligands, macrophage inflammatory protein (MIP)-1 α and MIP-1 β (refs 7, 8). The apparent affinity of the interaction between gp120 and CCR-5 was dramatically lower in the absence of soluble CD4. Additionally, in the absence of gp120,

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an interaction between a two-domain CD4 fragment and CCR-5 was observed. A gp120 fragment retaining the CD4-binding site and overlapping epitopes was able to interact with CCR-5 only if the V3 loop, which can specify HIV-1 tropism and chemokine receptor choice^{2,9-11}, was also present on the molecule. Neutralizing antibodies directed against either CD4-induced or V3 epitopes on gp120 blocked the interaction of gp120-CD4 complexes with CCR-5. These results suggest that HIV-1 attachment to CD4 creates a high-affinity binding site for CCR-5, leading to membrane fusion and virus entry.

The ability of purified gp120 exterior envelope glycoproteins from macrophage-tropic primary or laboratory-adapted HIV-1 to compete with the natural ligands for CCR-5 was studied in the

absence and presence of soluble forms of CD4¹². Radiolabelled MIP-1 α , MIP-1 β and RANTES, but not MCP-1 or interleukin (IL)-8, bound to CCR5F-L1.2 cells, which are murine lymphocytes stably expressing an epitope-tagged CCR-5 protein (5×10^4 to 8×10^4 binding sites per cell), with dissociation constants of 1.1, 0.4 and 0.2 nM, respectively (data not shown). The HIV-1 gp120 derivatives used in this study (Fig. 1a) were derived from the JR-FL, BAL and YU2 macrophage-tropic primary viruses or from the HXBc2 laboratory-adapted virus. Some of the gp120 glycoproteins contain deletions of the first (C1) and fifth (C5) conserved regions, which are important for the gp120 interaction with the gp41 transmembrane glycoprotein¹³, or deletions of the major V1/V2 or V3 variable loops^{14,15}. All of the gp120 derivatives used in

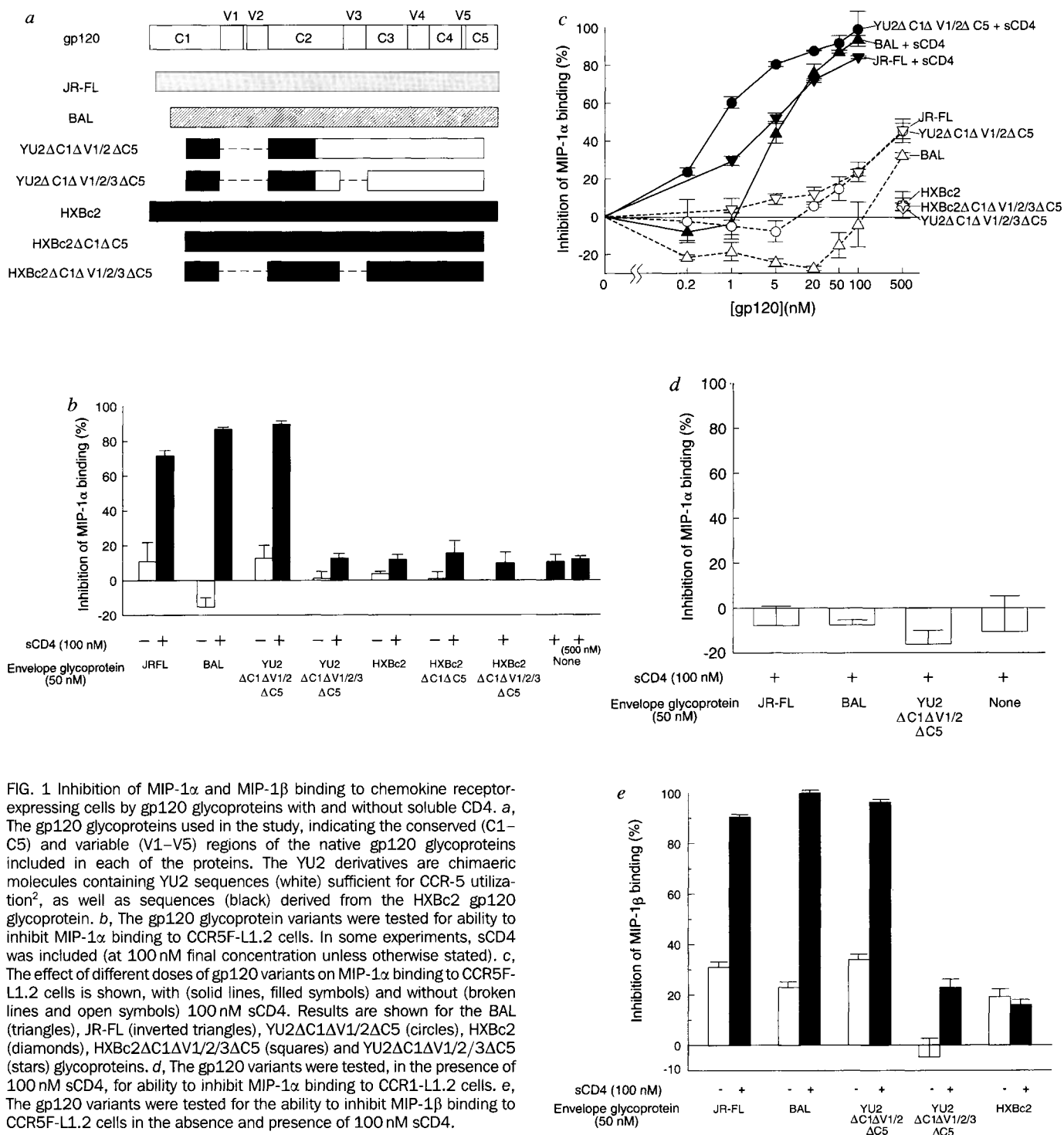


FIG. 1 Inhibition of MIP-1 α and MIP-1 β binding to chemokine receptor-expressing cells by gp120 glycoproteins with and without soluble CD4. **a**, The gp120 glycoproteins used in the study, indicating the conserved (C1-C5) and variable (V1-V5) regions of the native gp120 glycoproteins included in each of the proteins. The YU2 derivatives are chimeric molecules containing YU2 sequences (white) sufficient for CCR-5 utilization², as well as sequences (black) derived from the HXBc2 gp120 glycoprotein. **b**, The gp120 glycoprotein variants were tested for ability to inhibit MIP-1 α binding to CCR5F-L1.2 cells. In some experiments, sCD4 was included (at 100 nM final concentration unless otherwise stated). **c**, The effect of different doses of gp120 variants on MIP-1 α binding to CCR5F-L1.2 cells is shown, with (solid lines, filled symbols) and without (broken lines and open symbols) 100 nM sCD4. Results are shown for the BAL (triangles), JR-FL (inverted triangles), YU2 Δ C1 Δ V1/2 Δ C5 (circles), HXBc2 (diamonds), HXBc2 Δ C1 Δ V1/2/3 Δ C5 (squares) and YU2 Δ C1 Δ V1/2/3 Δ C5 (stars) glycoproteins. **d**, The gp120 variants were tested, in the presence of 100 nM sCD4, for ability to inhibit MIP-1 α binding to CCR1-L1.2 cells. **e**, The gp120 variants were tested for the ability to inhibit MIP-1 β binding to CCR5F-L1.2 cells in the absence and presence of 100 nM sCD4.

this study have been shown to bind CD4 efficiently, with dissociation constants of 4–30 nM, and to bind the F105 and 17b antibodies in the absence and presence of soluble CD4, respectively^{14–16} (R.W. and J.S., manuscript in preparation). The F105 antibody recognizes a discontinuous HIV-1 gp120 epitope that overlaps the CD4 binding site¹⁷, and the 17b antibody binds a discontinuous gp120 epitope that is increased in exposure following CD4 binding¹⁸. Two soluble forms of the CD4 glycoprotein were included in the study: four-domain soluble CD4 (sCD4) and a protein consisting of the amino-terminal two domains of CD4 (D1D2 sCD4) (ref. 12). In the presence of sCD4, the JR-FL, BAL and YU2AC1ΔV1/2ΔC5 glycoproteins, which were derived from the macrophage-tropic primary viruses, significantly inhibited MIP-1α binding to CCR5F-L1.2 cells (Fig. 1b). A dose-response curve indicated half-maximal inhibitory concentrations (IC_{50}) of 4.7, 5.5 and 0.7 nM for the JR-FL, BAL and YU2AC1ΔV1/2ΔC5 glycoproteins, respectively, in the presence of sCD4 (Fig. 1c). In the absence of sCD4, 500 nM concentrations of the JR-FL, BAL and YU2AC1ΔV1/2ΔC5 glycoproteins inhibited 32–45% of MIP-1α binding to CCR5F-L1.2 cells. For these gp120 derivatives, the presence of sCD4 resulted in a 100 to 1,000-fold increase in the efficiency of the observed inhibition.

In contrast to the above results, the YU2AC1ΔV1/2/3ΔC5 glycoprotein, which differs from the YU2AC1ΔV1/2ΔC5 glycoprotein in having no gp120 V3 loop, was dramatically reduced in the ability to inhibit MIP-1α binding in the presence and absence of sCD4 (Fig. 1b, c). Thus the V3 loop seems to be essential for the inhibition of MIP-1α binding to the CCR5F-L1.2 cells. No significant inhibition of MIP-1α binding was observed for any of the HXBc2 envelope glycoprotein derivatives over that seen with sCD4 alone. The latter inhibition was 12% or less, and did not increase much at higher sCD4 concentrations. The inhibition of MIP-1α binding by the JR-FL, BAL and YU2AC1ΔV1/2ΔC5 proteins was specific for CCR5F-L1.2 cells, as no significant inhibition of MIP-1α binding to L1.2 cells expressing CCR-1 was observed for these envelope glycoproteins, even when sCD4 was

present (Fig. 1d). Similarly, no inhibition of MIP-1α binding to THP-1 cells was seen for these gp120 glycoprotein-sCD4 mixtures (data not shown). The THP-1 cells used in these experiments specifically bound MIP-1α, but not MIP-1β, indicating that CCR-5 is not efficiently expressed on the cell surface (L.W., unpublished data). The observed chemokine-receptor specificity indicates that interaction with MIP-1α is not the basis for the observed inhibition of binding by the gp120 glycoprotein-CD4 complexes. The JR-FL, BAL and YU2AC1ΔV1/2ΔC5 glycoproteins, but not the YU2AC1ΔV1/2/3ΔC5 or the HXBc2ΔC1ΔC5 glycoproteins, also inhibited MIP-1β binding to CCR5F-L1.2 cells in the presence of sCD4 (Fig. 1e). Together these results indicate that MIP-1α and MIP-1β binding to CCR-5-expressing cells can be specifically inhibited by gp120 glycoproteins or some gp120 fragments derived from macrophage-tropic, primary HIV-1 isolates; that the efficiency of this inhibition is dramatically increased in the presence of soluble CD4; and that an intact V3 loop seems to be important for the effect.

The two-domain soluble CD4 (D1D2 sCD4) was compared with four-domain sCD4 for the ability to promote the high-affinity interaction with CCR-5 when mixed with gp120 glycoproteins from macrophage-tropic primary HIV-1. Mixtures of these gp120 glycoproteins with both D1D2 sCD4 and sCD4 efficiently inhibited MIP-1α and MIP-1β binding to CCR5F-L1.2 cells (Fig. 2a, b). In contrast to the sCD4 protein, D1D2 sCD4 inhibited MIP-1α and MIP-1β binding with an IC_{50} of 20–30 nM in the absence of the gp120 glycoprotein. This inhibitory effect of D1D2 sCD4 exhibited chemokine receptor specificity, as the D1D2 sCD4 did not efficiently inhibit MIP-1α binding to CCR1-L1.2 cells (Fig. 2c). Soluble VCAM, another soluble immunoglobulin family protein, had no significant effect on MIP-1α binding to CCR5F-L1.2 cells (Fig. 2a).

We examined the effect of anti-gp120 monoclonal antibodies on the inhibition of MIP-1α binding to CCR5F-L1.2 cells by the JR-FL gp120 glycoprotein in the presence of sCD4 (Fig. 3). All of the antibodies included in this study have previously been shown

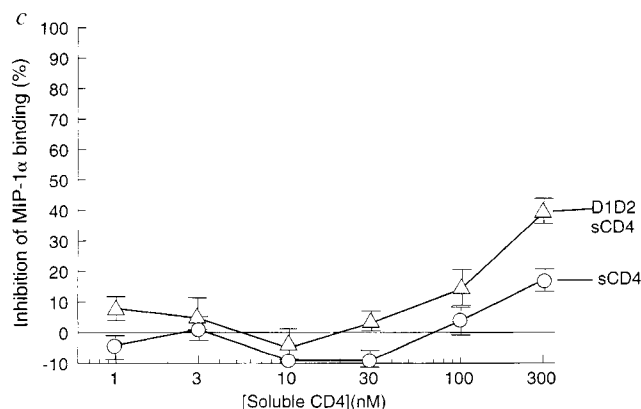
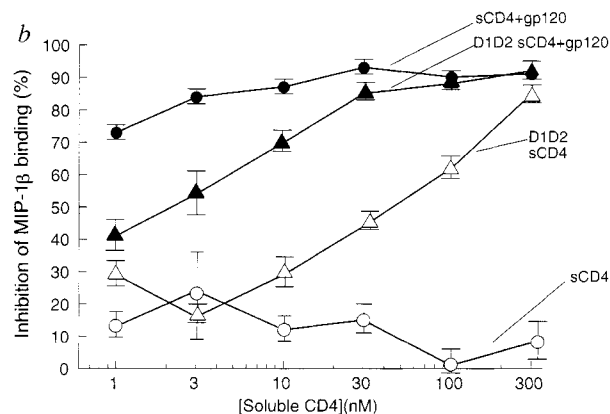
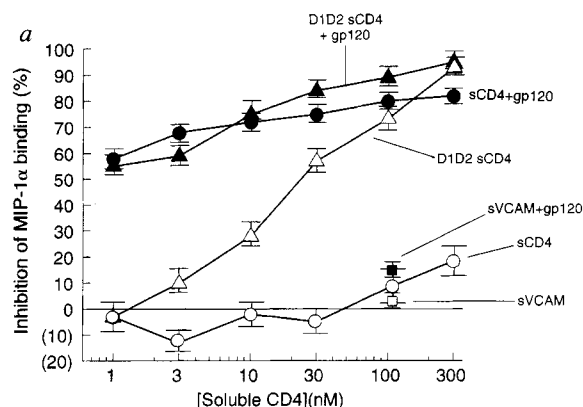


FIG. 2 Comparison of two-domain and four-domain soluble CD4 proteins for ability to inhibit MIP-1α and MIP-1β binding with and without gp120 glycoproteins. a, The ability of D1D2 sCD4 (triangles), sCD4 (circles) and soluble VCAM (squares) to inhibit MIP-1α binding to CCR5F-L1.2 cells with (filled symbols) and without (open symbols) 50 nM BAL gp120. b, The ability of D1D2 sCD4 (triangles) and sCD4 (circles) to inhibit MIP-1β binding to CCR5F-L1.2 cells with (filled symbols) and without (open symbols) 50 nM YU2AC1ΔV1/2ΔC5 glycoprotein. c, The D1D2 sCD4 (triangles) and sCD4 (circles) proteins were tested for ability to inhibit MIP-1α binding to CCR1-L1.2 cells at the indicated concentrations, in the absence of gp120 glycoproteins.

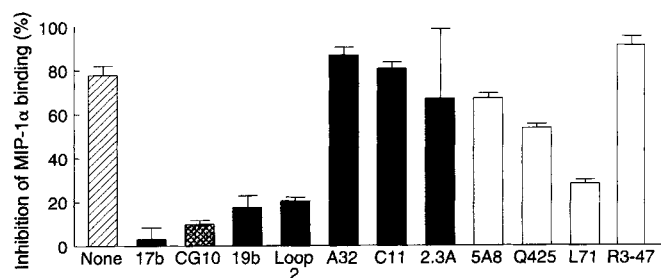


FIG. 3 Effects of monoclonal antibodies on the inhibition of MIP-1 α binding to CCR-5-expressing cells by mixtures of gp120 and sCD4. Monoclonal antibodies (final concentration 500 nM) directed against gp120 (black), CD4 (white) or against hybrid gp120-CD4 epitopes (cross-hatched) were tested for the ability to affect the inhibition of MIP-1 α binding to CCR5F-L1.2 cells by a mixture of the JR-FL gp120 glycoprotein (50 nM final concentration) and sCD4 (100 nM final concentration). The inhibition of MIP-1 α binding to CCR5F-L1.2 cells by the JR-FL gp120-sCD4 mixture in the absence of added antibody is also shown (hatched).

to recognize gp120 in the presence of sCD4 (refs 15, 18–20) and were able to precipitate the JR-FL gp120 glycoprotein (data not shown). The 17b neutralizing antibody, which recognizes a discontinuous, conserved gp120 epitope exposed better after CD4 binding¹⁸, blocked the inhibition of MIP-1 α binding by the gp120-sCD4 mixture. Inhibition of MIP-1 α binding was also decreased by the addition of the CG10 antibody, which recognizes an epitope present only on gp120-CD4 complexes²⁰. Two neutralizing antibodies, 19b and loop 2, which recognize the V3 loop of the JR-FL gp120 glycoprotein²¹, significantly blocked the effect of the JR-FL gp120-CD4 mixture on MIP-1 α binding. In contrast to the above results, non-neutralizing antibodies (A32, C11, 2.3A) that recognize gp120 domains interacting with the gp41 transmembrane glycoprotein¹⁹ did not block the ability of gp120-sCD4 complexes to inhibit MIP-1 α binding. Of the anti-CD4 antibodies tested (5A8, Q425, R3-47 and L71)^{22–25}, only L71, which can decrease gp120-CD4 interaction in some contexts²⁴, moderately affected the observed MIP-1 α inhibition.

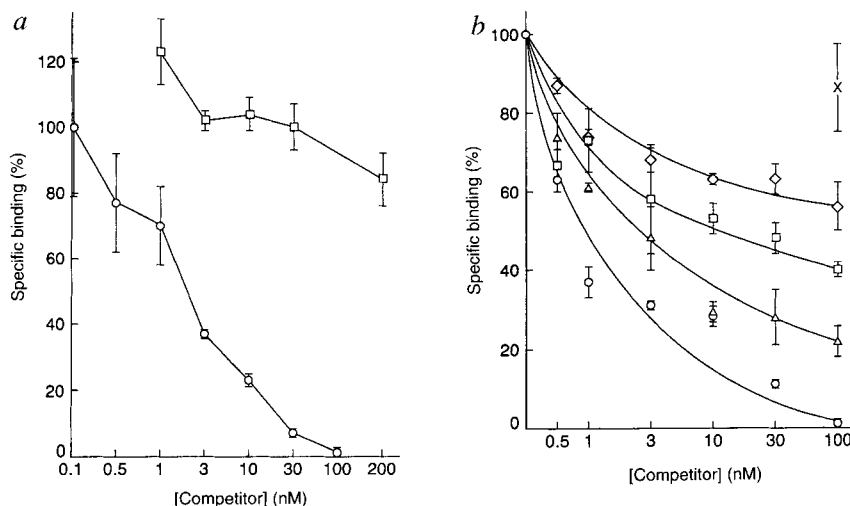
The direct binding of a radiolabelled macrophage-tropic virus gp120 protein, YU2AC1 Δ V1/2 Δ C5, to CCR5F-L1.2 cells was studied in the presence of sCD4. The specific binding of the labelled YU2AC1 Δ V1/2 Δ C5 protein to CCR5F-L1.2 cells was efficiently inhibited by the YU2AC1 Δ V1/2 Δ C5 protein but not by the HXBc2 Δ C1 Δ C5 protein (Fig. 4a). Scatchard analysis revealed that the YU2AC1 Δ V1/2 Δ C5 binding occurred with a dissociation

constant of 4–6 nM and that the number of binding sites on the CCR5F-L1.2 cells was similar for the YU2AC1 Δ V1/2 Δ C5 protein and for MIP-1 β (data not shown). RANTES, MIP-1 α and MIP-1 β decreased the binding of the YU2AC1 Δ V1/2 Δ C5 protein to CCR5F-L1.2 cells, whereas the V3 loop-deleted protein, YU2AC1 Δ V1/2/3 Δ C5, did not (Fig. 4b). These results indicate that the natural CCR-5 ligands inhibit the binding of a macrophage-tropic HIV-1 gp120-sCD4 complex to CCR-5-expressing cells.

We have shown that the gp120 glycoproteins derived from macrophage-tropic, primary HIV-1 interact specifically with CCR-5 and inhibit MIP-1 α or MIP-1 β binding to transfected cells that express CCR-5. The gp120-CCR-5 interaction was dramatically enhanced by CD4, suggesting that a major consequence of CD4 binding is, in addition to virus attachment to the target cell, promotion of subsequent events, such as chemokine receptor binding, that are important for the membrane fusion process. CD4-mediated induction of CCR-5 binding may contribute to the observed enhancement of primary HIV-1 infection by sCD4 (ref. 26). A sequential, two-step process for viral attachment and entry allows conserved elements on the viral glycoproteins interacting with chemokine receptors to remain sequestered from antiviral antibodies, until such time as proximity to the target cell membrane is achieved by the virus. The limited accessibility of antibodies to CD4-induced gp120 moieties in the context of the membrane-anchored, oligomeric envelope glycoprotein-CD4 complex may then allow membrane fusion and virus entry to proceed despite the humoral immune response.

A low-affinity interaction with CCR-5 can apparently occur in the absence of CD4 for gp120 variants derived from macrophage-tropic primary HIV-1. These results indicate that there is a major site for CCR-5 interaction on the gp120 glycoprotein. The CCR-5-interactive region must be quite well-conserved, because CCR-5 can be used as a co-receptor by diverse HIV-1 strains, as well as by simian immunodeficiency viruses² (L. Marcon *et al.*, submitted). Although additional studies will be required to define this site precisely, several insights into the CCR-5-interactive region are provided by our data. First, the CCR-5-interactive region is preserved on a gp120 fragment lacking the C1, V1/V2 and C5 regions. Second, some HIV-1-neutralizing antibodies that do not interfere with gp120-CD4 binding blocked the interaction of soluble CD4-gp120 complexes with CCR-5. One of these antibodies, 17b, recognizes a discontinuous gp120 epitope that is exposed better upon CD4 binding¹⁸, a property shared by the CCR-5-interactive region. Loop 2 and 19b, other antibodies inhibiting CCR-5 interaction, are directed against the gp120 V3 loop²¹. The 17b and V3 epitopes themselves cannot represent the CCR-5-interactive region on gp120, because natural primate immunodeficiency viruses that are not recognized by the 17b,

FIG. 4 Binding of a radiolabelled macrophage-tropic primary virus gp120 derivative to CCR-5-expressing cells. a, With 100 nM sCD4, the binding of iodinated YU2AC1 Δ V1/2 Δ C5 protein to CCR5F-L1.2 cells was determined in the presence of increasing concentrations of either YU2AC1 Δ V1/2 Δ C5 protein (circles) or HXBc2 Δ C1 Δ C5 protein (squares). b, With 100 nM sCD4 and 100 nM HXBc2 Δ C1 Δ C5 protein, the binding of iodinated YU2AC1 Δ V1/2 Δ C5 protein to CCR5F-L1.2 cells was measured in the presence of the indicated concentrations of YU2AC1 Δ V1/2 Δ C5 protein (circles), MIP-1 α (diamonds), MIP-1 β (squares), RANTES (triangles) or YU2AC1 Δ V1/2/3 Δ C5 protein (cross).



19b and loop 2 antibodies can use CCR-5 as a co-receptor² (L. Marcon, submitted). However, the CCR-5–gp120 interaction is likely to involve gp120 structures proximal to these epitopes. Third, deletion of the V3 loop, which can determine HIV-1 tropism and chemokine receptor use^{2,9–11}, disrupted CCR-5 interaction. A component of the chemokine-receptor binding site on gp120 may reside in a V3 structure demonstrating moderate variability. Together these observations implicate a discontinuous gp120 structure in the vicinity of the 17b epitope, with a probable contribution from V3 sequences, as the CCR-5-interactive moiety on the gp120 glycoprotein. The proximity of the 17b epitope and the V3 loop on the native gp120 glycoprotein has been suggested by previous mutational and antibody-competition analyses^{15,18,19}.

Our results suggest that an interaction between D1D2 sCD4 and CCR-5 occurs in the absence of gp120 glycoproteins. Because full-length sCD4 did not compete for MIP-1 α and MIP-1 β binding to CCR-5-expressing cells at comparable concentrations, the D1D2 sCD4 seems to expose a CCR-5-interactive region more efficiently. That a CD4 fragment spontaneously and specifically binds CCR-5 raises the possibility that native CD4 sequences contribute directly to a high-affinity binding site for CCR-5 in the presence of the appropriate gp120 glycoprotein. If the observed D1D2 sCD4–CCR5 interaction is biologically relevant, it is probably not restricted to CCR-5, because other chemokine receptors can be used as co-receptors for HIV-1 variants^{1–3}. The regions of gp120 and CD4 that potentially contribute to CCR-5 interaction are expected to be quite distant from the target cell membrane, at least when the virus initially attaches to CD4. This implies that significant conformational rearrangements of envelope glycoprotein–CD4 complexes may occur during the course of HIV-1 entry to optimise CCR-5 binding. An improved understanding of the interactive regions and mechanisms should facilitate therapeutic and prophylactic strategies targeting HIV-1 entry. □

Methods

Cells. The murine pre-B-lymphoma line, L1.2, was stably transfected with CCR-5 cDNA, and tagged at the N terminus with a FLAG epitope (Kodak), in the pMRB101 expression vector, as described²⁷. The pMRB101 plasmid is a derivative of Ee6hcmvbgllii that contains the *Escherichia coli* gene *gpt* and was provided by M. Robinson (CellTech). The cell-surface expression of CCR-5 was monitored by staining with an anti-FLAG antibody, and cells with a high level of CCR-5 expression were selected by several rounds of limiting dilution and rescreeing. L1.2 cells stably expressing CCR-1 were provided by J. Campbell and E. Butcher (Stanford University). Scatchard analysis of MIP-1 α binding to CCR1-L1.2 cells indicated a dissociation constant of 8 nM and 2×10^4 binding sites per cell (S. Qin, unpublished results).

Chemokine binding and competition assays. ¹²⁵I-labelled human MIP-1 α and MIP-1 β and unlabelled chemokine were purchased from DuPont NEN (Boston, MA) and Peprotech (Rocky Hill, NJ), respectively. CCR5F-L1.2 cells were washed and resuspended in binding buffer (50 mM HEPES, pH 7.5, 1 mM CaCl₂, 5 mM MgCl₂ and 0.5% BSA) at a concentration of 5×10^5 cells ml⁻¹. For binding and competition studies, which were conducted in a final volume of 100 μ l, gp120 glycoproteins were mixed with soluble CD4 on ice for 5–10 min, after which monoclonal antibodies were added if appropriate. After another 5–10 min, 25 μ l of cell suspension (1.25×10^5 cells) was added, followed by radiolabelled chemokine (final concentration 0.1 nM). The reactions were then incubated at 37 °C for 30–45 min and stopped by transferring the mixture to GFB filter plates, which were washed twice with binding buffer containing 0.5 M NaCl. The plates were dried, immersed in MicroScint scintillation fluid and counted. The total binding was determined by including only radiolabelled chemokine and cells in the reaction. Nonspecific (background) binding was determined in the presence of 100 nM unlabelled chemokine. The percentage of inhibition was calculated using the formula: % inhibition ligand binding = $100 - 100(S - B)/(T - B)$, where *S* is the test sample, *B* is the background and *T* is the total binding. Each experiment was performed twice with duplicates.

Recombinant glycoproteins. All of the gp120 glycoproteins were produced from stably transfected *Drosophila* Schneider 2 cells, using the pMt vector and selectable marker, pc hygro²⁸. The JR-FL and BAL gp120 proteins have been described¹⁶ and were provided by R. Sweet (SmithKline Beecham). The BAL gp120 derivative contains an N-terminal deletion of 32 residues compared with the wild-type gp120 glycoprotein. The YU2 glycoproteins were produced from a chimaeric *env* gene, containing YU2 sequences from BgIII (nucleotide 6,620) to BgIII (nucleotide 7,200) in an HXBc2 background. The Δ C1 proteins contain

deletions up to and including residue 82. The Δ V1/2 and Δ V3 proteins contain deletions equivalent to the Δ 128–194 and Δ 298–329 deletions, respectively, previously described for the HXBc2 glycoprotein¹⁵. The Δ C5 proteins contain a deletion of the C-terminal 19 residues of the mature gp120 glycoprotein. All gp120 proteins use the tissue plasminogen activator signal sequence for translocation into the endoplasmic reticulum. Protein expression of recombinant YU2 and HXBc2 derivatives was induced by transfer of *Drosophila* lines into serum-free medium containing 750 mM CuSO₄ for 7 days at 25 °C. Recombinant proteins were purified by passage of cell supernatants over an F105 monoclonal antibody column, which was extensively washed with PBS containing 500 mM NaCl and then re-equilibrated in PBS containing 150 mM NaCl. The gp120 glycoproteins were eluted with 100 mM glycine-HCl, pH 2.8, and fractions were immediately neutralized with 1 M Tris base. The gp120 glycoproteins were concentrated using Centrprep 30 spin filters (Amicon), and resuspended in PBS containing protease inhibitors. Protein concentrations were determined by comparison with commercially available gp120 (Agmed) on Coomassie blue-stained SDS–PAGE gels. All of the gp120 preparations were homogeneous, with the exception of the BAL gp120 preparation, in which approximately 5% of the protein was proteolytically cleaved. Soluble CD4 proteins¹² were provided by R. Sweet (SmithKline Beecham). The soluble VCAM protein used in these studies is a chimaera of human D1D2 VCAM and the murine constant κ chain. The soluble VCAM was expressed in SF9 cells by a recombinant baculovirus and purified on a protein A column.

Gp120 binding assay. The YU2 Δ C1 Δ V1/2 Δ C5 protein was iodinated to a specific activity of 900 Ci mmol⁻¹ using solid-phase lactoperoxidase and glucose oxidase (Enzymobeads from BioRad, Richmond, CA)²⁹. The CCR5F-L1.2 cells were preincubated for 10–20 min at room temperature in phosphate-buffered saline. The labelled protein was then added (final concentration 0.1 nM) to 5×10^5 cells in duplicate in 100 μ l of 50 mM HEPES, pH 7.2, containing 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA, 100 nM sCD4 and different concentrations of unlabelled YU2 Δ C1 Δ V1/2 Δ C5 or HXBc2 Δ C1 Δ C5 protein. For chemokine inhibition, the iodinated YU2 Δ C1 Δ V1/2 Δ C5 protein was incubated with the CCR5F-L1.2 cells in the same buffer containing 100 nM sCD4, 100 nM HXBc2 Δ C1 Δ C5 and different concentrations of RANTES, MIP-1 α , MIP-1 β or the YU2 Δ C1 Δ V1/2 Δ C5 protein. After 30 min incubation at 37 °C, cells were washed in 50 mM HEPES, pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA and 0.5 M NaCl and bound radioactivity counted. The background (nonspecific) binding was determined in the presence of 100 nM unlabelled YU2 Δ C1 Δ V1/2 Δ C5 protein. The specific binding was calculated using the formula: % specific binding = $100(S - B)/(T - B)$, where *S* is the observed counts bound at a given concentration of unlabelled competitor protein, *T* is the observed counts bound in the absence of competitor, and *B* is the background count.

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CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5

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The β -chemokine receptor CCR-5 is an essential co-factor for fusion of HIV-1 strains of the non-syncytium-inducing (NSI) phenotype with CD4⁺ T-cells¹⁻⁵. The primary binding site for human immunodeficiency virus (HIV)-1 is the CD4 molecule, and the interaction is mediated by the viral surface glycoprotein gp120 (refs 6, 7). The mechanism of CCR-5 function during HIV-1 entry has not been defined, but we have shown previously that its β -chemokine ligands prevent HIV-1 from fusing with the cell¹. We therefore investigated whether CCR-5 acts as a second binding site for HIV-1 simultaneously with or subsequent to the interaction between gp120 and CD4. We used a competition assay based on gp120 inhibition of the binding of the CCR-5 ligand, macrophage inflammatory protein (MIP)-1 β , to its receptor on activated CD4⁺ T cells or CCR-5-positive CD4⁺ cells. We conclude that CD4 binding, although not absolutely necessary for the gp120-CCR-5 interaction, greatly increases its efficiency. Neutralizing monoclonal antibodies against several sites on gp120, including the V3 loop and CD4-induced epitopes, inhibited the interaction of gp120 with CCR-5, without affecting gp120-CD4 binding. Interference with HIV-1 binding to one or both of its receptors (CD4 and CCR-5) may be an important mechanism of virus neutralization.

MIP-1 β is the most specific ligand for CCR-5 (refs 8-10) because MIP-1 α and RANTES also bind with high affinity to other members of the β -chemokine receptor family on lymphoid cells⁸⁻¹¹. We therefore used MIP-1 β as the CCR-5 ligand in the competition assays. In common with other members of this receptor family¹², CCR-5 is a mitogen-response gene. Its expression in quiescent, purified CD4⁺ T-cells is usually minimal, but 3 days after activation of the cells by phytohaemagglutinin and interleukin (IL)-2, we observed strong increases in CCR-5 messenger RNA and ¹²⁵I-labelled-MIP-1 β binding (data not shown). As specificity controls, we used CD4⁺ T cells from individuals homozygous for defective CCR-5 alleles^{13,14}. The amount of specific (that is, cold MIP-1 β -competed) ¹²⁵I-MIP-1 β (0.1 nM) binding to cells from three such individuals was 92 ± 12 c.p.m. per 2×10^6 cells (mean $\pm$ s.d.). In contrast, mean binding to cells from 21 control individuals was $1,044 \pm 1,073$ c.p.m. per 2×10^6 cells (range, 222-4,846 c.p.m.). Most of the ¹²⁵I-MIP-1 β reactivity with activated CD4⁺ T cells therefore derives from binding to CCR-5.

When recombinant, monomeric gp120s were added with ¹²⁵I-MIP-1 β to activated CD4⁺ T cells, we found that gp120 from the NSI strain JR-FL (which used CCR-5 for entry¹) strongly inhibited MIP-1 β binding (Fig. 1a; Table 1). Half-maximal inhibition occurred in the range $0.1-1.0 \mu\text{g ml}^{-1}$ (0.8-8 nM) gp120, which is similar to the association constant for the gp120-CD4 interaction⁷. In contrast, gp120 from the T-cell-line adapted (TCLA) SI strain LAI was ineffective (Fig. 1a; Table 1). This virus uses fusin (CXCR-4), but not CCR-5, for entry¹⁻⁵. Mutants of JR-FL and LAI gp120s which lack the V3 loop (Δ -V3 gp120), but bind CD4 with high affinity, did not block MIP-1 β binding (Fig. 1a). However, peptides (15-residue if not specified) from the V3 loops of the following strains were also inactive: JR-FL (32-residue), RA, VS, Case-B (each NSI); HxB2, MN, SF-2 (each TCLA) (peptides were added at $1 \mu\text{g ml}^{-1}$, the approximate molar equivalent of $60 \mu\text{g ml}^{-1}$ gp120). An oligomeric complex of JR-FL gp120 non-covalently associated with the ectodomain of gp41 was an effective inhibitor of MIP-1 β binding, but a recombinant molecule comprising only the gp41 ectodomain was not (Table 1), although the latter molecule may not fold into a native structure¹⁵.

HIV-1 strains from genetic subtypes A, B, C and E can use CCR-5 for entry³, and we have found that MIP-1 β inhibits the replication of most primary, NSI HIV-1 strains from subtypes A to E. This breadth of reactivity of HIV-1 with CCR-5 extended to the

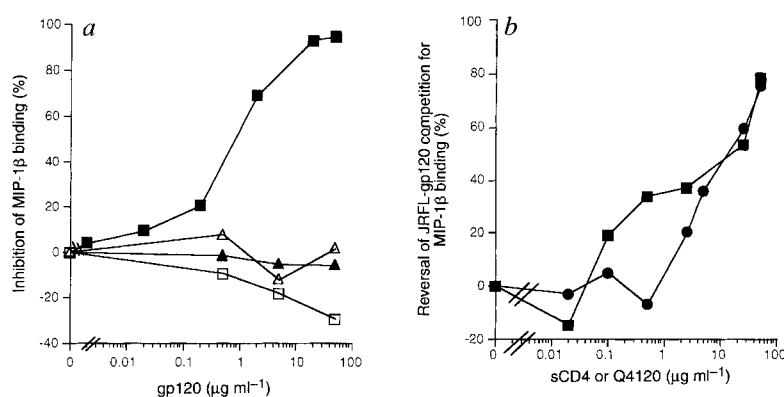
TABLE 1 Effect of recombinant gp120 on MIP-1 β binding

	gp120 ($\mu\text{g ml}^{-1}$)						V3 sequence
	0.1	0.2	0.5	5	20	50	
NSI gp120							
JR-FL (B)	44 $\pm$ 9	40 $\pm$ 23	58 $\pm$ 26	67 $\pm$ 9	91 $\pm$ 3	95 $\pm$ 6	CTRPNNNTRKSIHIGPGRAFYTGTGEIGDIRQAHC
JR-FL (B)*	46 $\pm$ 28	56 $\pm$ 3	84 $\pm$ 11				
SF 162 (B)	81 $\pm$ 8	113 $\pm$ 41					CTRPNNNTRKSIITIGPGRAFYTGTGDIIGDIRQAHC
W61D (B)	39 $\pm$ 14	53 $\pm$ 17	65 $\pm$ 23		86 $\pm$ 12		CTRPNNNTRKGIHIGPGRAFYAARKIIGDIRQAHC
92TH014 (B)	6 $\pm$ 1	57 $\pm$ 16	65 $\pm$ 5				CTRPNNNTRKSIHLGPGRAWYTTGGIIGDIRQAHC
SF 170 (A)	87 $\pm$ 45	140 $\pm$ 49					CTRPNNNTRKSVRIGPGQAFYATGDIIGDIRQAYC
92RW020 (A)*	21 $\pm$ 39	49 $\pm$ 22	74 $\pm$ 36				CTRPNNNTRKGVRIIGPGQAFYTGDIIGDIRQAHC
CM243 (E)	17 $\pm$ 14	40 $\pm$ 11	56 $\pm$ 38		76 $\pm$ 24		CTRPSNNTRPSITVGPQGVFYRTGDIIGDIRRAYC
TCLA gp120							
LAI (B)			-1 $\pm$ 3	-5 $\pm$ 36	-18 $\pm$ 39	-6 $\pm$ 9	CTRPNNNTRKSIRIQRGPGRAFVTGKIGNMRQAHC
MN (B)			-20 $\pm$ 14	-23 $\pm$ 4	1 $\pm$ 0	0 $\pm$ 6	CTRPNNNTRKRIHIGPGRAFYTGTGNIIGDIRQAHC
SF-2 (B)			-1 $\pm$ 4	3 $\pm$ 3	8 $\pm$ 26	21 $\pm$ 3	CTRPNNNTRKSIYIGPGRAFHTTGRIGDIRKAHC
gp41							
IIIB (B)				24 $\pm$ 5			

Recombinant proteins were titrated in the presence of 0.1 nM ¹²⁵I-MIP-1 β and added to activated CD4⁺ T cells. Percentage inhibitions of ¹²⁵I-MIP-1 β binding at each gp120 concentration are shown, and are the means $\pm$ s.d. of 2-4 independent experiments. No value indicates that the gp120 molecule was not tested at that concentration (several molecules were not available at concentrations $>1 \mu\text{g ml}^{-1}$).

*An oligomeric gp120/gp41 complex.

FIG. 1 CD4-dependent competition between gp120 and MIP-1 β for CCR-5 binding. **a**, JR-FL gp120 (filled squares), LAI gp120 (filled triangles), JR-FL- Δ V3 gp120 (open squares) or LAI- Δ V3 gp120 (open triangles) was added to activated CD4⁺ T cells and the extent of specific ¹²⁵I-MIP-1 β binding determined. Data shown are the means of three independent experiments, each performed in duplicate. **b**, JR-FL gp120 (2 μ g ml⁻¹) and ¹²⁵I-MIP-1 β (0.1 nM) were added to activated CD4⁺ T cells in the presence of the monoclonal antibody Q4120 (filled circles) or sCD4 (filled squares) at the concentrations indicated. The extent of specific ¹²⁵I-MIP-1 β binding was determined, and the percentage inhibition of the gp120 competitive effect was calculated for each antibody concentration (none present is 0% inhibition). The experiment shown was one of two performed, each yielding similar results.



¹²⁵I-MIP-1 β competition assay. Recombinant gp120s from the following NSI primary strains were competitive, with half-maximal inhibition of MIP-1 β binding occurring at concentrations around 0.1–0.5 μ g ml⁻¹: JR-FL (subtype B), SF162 (B), W61D (B, 92TH014 (B), SF170 (A), 92RW020 (A) and CM243 (E) (Table 1). In contrast, no competition was observed with gp120s from the TCLA subtype B strains LAI, MN and SF-2 (Table 1), although each could bind CD4 with high affinity (not shown). Thus the phenotype of the virus from which a gp120 molecule is derived is more important than the viral genotype in determining interactions with CCR-5.

We assessed the role of CD4 in the competition between NSI gp120 and MIP-1 β by using antagonists of the gp120–CD4 interaction. The monoclonal antibodies Q4120 and L77, which react with domain 1 of CD4 to inhibit gp120 binding¹⁶, and soluble CD4 (sCD4), which reacts with gp120 to inhibit CD4 binding¹⁷, both reversed the inhibition by JR-FL gp120 of MIP-1 β binding to CCR-5 (Fig. 1b; Table 2). Monoclonal antibodies to other domains of CD4 (which do not block gp120–CD4 binding¹⁶) were ineffective (Table 2) and, in the absence of gp120, sCD4 (50 μ g ml⁻¹) caused no inhibition of MIP-1 β binding (data not shown). An interaction with cell-surface CD4 is therefore impor-

TABLE 2 Monoclonal antibody inhibition of the gp120 interaction with CCR-5

	Epitope	Monoclonal antibody	Inhibition (%)	±s.d.
CD4 antibodies	CD4-D1	Q4120	83	27
		L77	77	12
	CD4-D2	5A8	3	15
	CD4-D3	Q425	15	23
	CD4-D4	L120	-17	23
anti-gp120 antibodies, non-neutralizing face	C5	D7324	-13	1
		23A	-11	14
	C1(D)	CRA-1	-2	18
		522-149	9	1
	C1(L)	133/192	8	7
		135/9	-2	7
	C1-C4	A32	51	11
	C1-C5	211c	5	11
anti-gp120 antibodies, neutralizing face	CD4bs	sCD4*	91	18
		15e†	65	18
	CD4bs	IgG1b12*	125	40
	C4(L)	G3 508†	57	1
	C4-V3 (D)	G3 42†	13	10
		48d*	54	33
	CD4i	17b*	79	41
	V2	697-D†	3	37
		SC258	-3	8
	V3	447-D*	109	2
		19b†	88	12
		83.1†	140	48
		2G12*	38	4

JR-FL gp120 (2 μ g ml⁻¹) inhibition of ¹²⁵I-MIP-1 β (0.1 nM) binding to activated CD4⁺ T cells was tested in the presence or absence of sCD4 (50 μ g ml⁻¹) or monoclonal antibodies CD4 (50 μ g ml⁻¹) or antibodies to gp120 (20 μ g ml⁻¹). Mean percentage reversals of the competitive effect of gp120 in the presence of each antibody (±s.d.; n = 2–4 independent experiments) are shown. The level of specific ¹²⁵I-MIP-1 β binding (c.p.m.) recorded in the presence of gp120 but the absence of antibody was set at 0%, and the level recorded in the absence of both gp120 and antibody was set at 100%. A negative percentage reversal indicates that the competitive effect of gp120 on ¹²⁵I-MIP-1 β binding was increased in the presence of the antibody. Also listed are the approximate positions of the antibody epitopes on gp120, as defined^{19,20}. References to the origin of the antibodies are described elsewhere^{19,20} or listed in the Methods section.

* Anti-gp120 monoclonal antibodies (or sCD4) able to neutralize HIV-1_{JR-FL}.

† Monoclonal antibodies with neutralizing activity against other HIV-1 strains (primary or TCLA).

tant for gp120 to interact efficiently with CCR-5 and block MIP-1 β binding. To determine whether CD4 was an absolute requirement, we prepared a stable human CD4⁺ CCR-5⁺ 293 cell line. These cells bind ¹²⁵I-MIP-1 β (specific binding up to 2,500 c.p.m. per 5×10^5 cells), whereas untransfected 293 cells do not (specific binding <50 c.p.m.). The binding of ¹²⁵I-MIP-1 β to the CD4⁺ CCR-5⁺ 293 cells was sporadically inhibited by JR-FL gp120, but only at the highest gp120 concentrations tested (50–100 $\mu\text{g ml}^{-1}$). The strongest competition observed on these cells was 73% inhibition of MIP-1 β binding by 50 $\mu\text{g ml}^{-1}$ of JR-FL gp120 (comparable inhibition was found in two other experiments), but competition was often not detected at all, and we never observed inhibition of MIP-1 β binding at lower concentrations of gp120. The addition of excess sCD4 to the CD4⁺ CCR-5⁺ cells neither reduced nor increased the inhibitory effect of JR-FL gp120 (data not shown).

The interaction between JR-FL gp120 and CCR-5 requires at least 100-fold higher gp120 concentrations on CD4⁺ cells than on CD4⁻ cells. We suggest this is because binding of gp120 to CD4 on the cell surface increases the probability of a gp120–CCR-5 interaction; either a gp120–CD4–CCR-5 ternary complex forms, or there are sequential interactions of gp120 with CD4, then CCR-5. One possibility is that the high-affinity association of gp120 with CD4 increases the probability of a lower-affinity interaction of gp120 with CCR-5 (a proximity effect). This is consistent with the finding that sCD4 does not substitute for cell-surface CD4, at least with JR-FL gp120. Alternatively, binding to CD4 may be necessary to (better) expose a CCR-5 binding site on gp120. This may be especially important in the context of virions, where some regions of the oligomeric envelope glycoproteins (including the V3 loop) that are accessible on monomeric gp120 are not optimally exposed before CD4 binding¹⁸.

To gain further insight into how the gp120–CD4 complex interacts with CCR-5 on activated CD4⁺ T cells, we used a panel of HIV-1 neutralizing and non-neutralizing anti-gp120 monoclonal antibodies^{19,20} having confirmed that each could bind to JR-FL gp120. The antibodies were tested for reversal of the competitive effect of gp120 on MIP-1 β binding (Table 2). As with sCD4, the antibodies to conformational (15e and IgG1b12) or linear (G3-508) epitopes overlapping the CD4-binding site²⁰ prevented JR-FL gp120 from competing with MIP-1 β . However, several antibodies that do not affect the binding of monomeric gp120 to CD4 (ref. 20) also inhibited the gp120–CCR-5 interaction (Table 2). These included three (447-D, 19b and 83.1) to the V3 loop; one (2G12) to a conserved epitope in the C3–V4 region; two (48d and 17b) to a conserved, CD4-induced epitope; and one (A32) to a second, CD4-induced epitope. All of these except A32 map to what we have defined as the gp120-neutralizing face²⁰. Eight other monoclonal antibodies that did not prevent JR-FL gp120 from blocking MIP-1 β binding (Table 2) cluster on what we have defined as the gp120-non-neutralizing face²⁰: their epitopes are accessible on monomeric gp120, but in the context of the oligomer they are occluded either by other gp120 subunits or by gp41 molecules^{19,20}. These ineffective antibodies include 2/11c to an epitope overlapping that of A32; for this reason, and because A32 neutralizes no HIV-1 strains strongly, the significance of the partial inhibitory action of A32 on the gp120–CCR-5 interaction is uncertain. Two monoclonal antibodies (697-D and SC258) to the V2 loop were also ineffective; although the V2 loop structure is modelled as being on (or above) the gp120 neutralizing face²⁰, these two antibodies are unable to neutralize HIV-1_{JR-FL}. The monoclonal antibodies 2G12, 17b, 447-D, 48d, IgG1b12, G3-508 and 697-D were also tested against the oligomeric JR-FL gp120/gp41 protein, and all except 697-D inhibited the interaction of this protein with CCR-5 (not shown).

Most of these antibodies to the neutralizing face of gp120 therefore either prevented gp120 from binding to CD4 or interfered with subsequent interactions with the CCR-5 second receptor. Not every antibody to this face of gp120 actually neutralizes HIV-1_{JR-FL}, as primary viruses resist neutralization,

and studies with recombinant proteins can only predict neutralization efficiencies imprecisely¹⁸. However, our findings may have implications for understanding how HIV-1 is neutralized by antibodies; blockade of the primary or secondary receptor interactions of the virus may be particularly important.

The simplest interpretation of the inability of Δ -V3 JR-FL gp120 to block MIP-1 β binding (Fig. 1a) is that the CCR-5 binding site is contained within the V3 loop. This would be consistent with the many observations that the V3 loop contains important determinants of HIV-1 phenotype and tropism^{18,21}, and can influence second-receptor usage². We believe, however, that the CCR-5 binding site is not limited to the V3 loop. The V3 sequences of gp120s of subtypes A, B and E that interact with CCR-5 are rather variable (Table 1). Furthermore, some simian immunodeficiency virus (SIV) strains can also use human CCR-5 as a second receptor (Z.-W. Chen and P. Marx, personal communication), but the V3 regions of HIV-1 and SIV have almost no primary sequence homology. Can all these sequences each form a binding site for the same, conserved cellular protein, when similar V3 sequences from TCLA HIV-1 strains cannot (Table 1)? Twin-site models of the interaction of ligands with chemokine receptors⁸ leave open the possibility that a relatively conserved section of the V3 loop could be one component of a multi-point binding site for CCR-5 on gp120. However, we suggest that the CCR-5 binding site must include a region of gp120 that is strongly conserved across the primate immunodeficiency viruses, not just across the HIV-1 genetic subtypes. Whether this is also the case for HIV-1 interactions with CXCR-4 remains to be determined.

The structure of the V3 loop may influence the nature of a complex binding site for CCR-5 on gp120. A region of gp120 overlapped by (but not necessarily identical to) the CD4-induced epitopes of monoclonal antibodies 48d and 17b is a good candidate for such a site. These antibodies recognize similar conformationally sensitive structures that are probably located around the bases of the V1, V2 and V3 loops^{22,23}. Deletion of the V3 loop from both HxBc2 gp120 and JR-FL gp120 destroys the 48d and 17b epitopes^{22,23} (J.M.B. and J.P.M. unpublished data), which may be relevant to the inability of the Δ -V3 JR-FL gp120 to interact with CCR-5 (Fig. 1a), and single amino-acid changes in the V3 and C4 regions of HIV-1_{LAI} also have major effects on the structure of these epitopes²⁴.

Further studies will be required to refine our understanding of the CCR-5 binding site. The efficiency with which β -chemokines inhibit the replication of NSI primary isolates in peripheral blood mononuclear cells (PBMCs) is dependent on strain but not subtype (A.T. and J.P.M., unpublished data), suggesting, perhaps, that the degree of overlap between the gp120 and β -chemokine binding sites on CCR-5 varies between gp120s. If so, the CCR-5 binding site on gp120 might be more flexible than the CD4 binding site. Finally, although sCD4 inhibited the interaction between JR-FL gp120 and CCR-5 on CD4⁺ cells, for some strains of HIV-1 and (especially) HIV-2 and SIV, sCD4 might enhance the efficiency of second-receptor interactions, and thereby facilitate the entry of these primate immunodeficiency viruses into CD4⁺ or CD4⁺ cells^{25,26}. □

Methods

Sources of reagents. Recombinant human β -chemokines were purchased from R&D Systems (Minneapolis) and ¹²⁵I-MIP-1 β (specific activity 2,200 Ci mmol⁻¹) was from Dupont-NEN. Anti-CD4 monoclonal antibodies were from Q. Sattentau²⁵, except for 5A8, from L. Burkly (Biogen)²⁷ and L120 (UK MRC AIDS Reagent Repository)¹⁶. Soluble CD4 has been described¹⁷. Monoclonal antibodies to gp120 were obtained from donors listed elsewhere^{19,20}, except for 23A (gp120 C terminus, from J. Robinson), 447-D and 697-D (ref. 28; Cellular Products Inc.) and 83.1 (ref. 29; Repligen). Recombinant gp41 (IIIB) ectodomain was from Viral Therapeutics Inc., and V3 peptides were obtained either from Repligen or the UK MRC AIDS Reagent Repository. Recombinant MN gp120 (Genentech), SF-2 gp120 (Chiron) and CM243 gp120 were provided by the NIAID AIDS Reagents Repository, and W61D gp120 (SmithKline Beecham, Belgium) was from the UK MRC AIDS Reagent Repository.

Recombinant, monomeric JR-FL and LAI gp120s, both full-length and with the V3-loops deleted, were expressed using vectors developed at Progenics Pharmaceuticals that contain a dihydrofolate reductase expression cassette. The expression of the gene is under the control of the cytomegalovirus immediate-early promoter. For Δ -V3 gp120s, the V3 loops were excised by the splicing-by-overlap-extension technique, such that the cysteines defining the loop were retained and spanned by the peptide sequence TGAGH. All constructs were sequenced to verify that no mutations were introduced during the cloning manipulations. The proteins were expressed in stably transfected Chinese hamster ovary cells (DBX-11), selected in nucleoside-free medium and amplified using methotrexate, following previously described methods¹⁷. The secreted proteins were purified to >95% homogeneity in a non-denaturing process comprising anion exchange, *Galanthus nivalis* lectin affinity and gel filtration chromatography. The purified proteins bound sCD4 with nanomolar affinity¹⁸.

For expression of SF162 gp160, a 3.5-kb *EcoRI*–*Bam*HI fragment containing the *env* gene was excised from the SV40-based vector pSM and subcloned into the *R1/Bgl*III sites of the β -actin-based expression vector pCAGGS. For expression of SF170 gp160, a 3.8-kb fragment containing the *env* gene was excised from the pBSKS⁺ plasmid, blunted by treatment with T4 DNA polymerase, and subcloned into the *RV/Xho*I sites of pCAGGS. The expression plasmids were transfected into 293 T cells by calcium phosphate co-precipitation. Soluble gp120 in the culture supernatant was collected after three days, filtered through 0.2- μ m filters and concentrated over an Amicon 1000 membrane.

The preparation of soluble, oligomeric forms of the JR-FL and 92RW020 envelope glycoproteins (and also monomeric gp120 from 92TH014) was as follows. The JR-FL *env* gene was provided by I. Chen (UCLA) and the *env* genes of 92TH014 and 92RW020 were obtained from the NIAID AIDS Reagent Repository³⁰. Soluble expression plasmids encoding gp120 and the gp41 ectodomain of JR-FL and 92RW020, or gp120 only of 92TH014, were constructed as described³⁰, and transfected into Chinese hamster ovary cells by the calcium phosphate method. The cleavage sites between the JR-FL and 92TH014 gp120 and gp41 moieties were retained, and proteins secreted as oligomers (J.A., J.M.B. and J.P.M., unpublished data). Envelope glycoproteins were partially purified from culture supernatants by immobilized metal-affinity chromatography. A control preparation, 93MW959(c), containing a gp120/gp41 molecule incompetent at CD4 binding, by virtue of a single point mutation at residue 457, did not compete with ¹²⁵I-MIP-1 β . The monomeric gp120 or oligomeric gp120/gp41 concentrations in unpurified culture supernatants were

estimated by denaturing the proteins¹⁹, then dot-blotting onto nitrocellulose membranes and detecting the gp120 with a cocktail of murine monoclonal antibodies to continuous epitopes¹⁹, followed by an anti-mouse IgG-HRP conjugate and the ECL chemiluminescence system (Amersham). Purified, monomeric JR-FL gp120 was used as a concentration standard¹⁷. The concentration of oligomeric gp120/gp41 complexes was defined as the total concentration of monomeric gp120 subunits in the preparation. High-affinity CD4 binding of the gp120s was confirmed by enzyme-linked immunosorbent assay (ELISA)¹⁹.

Cells and cell lines. PBMCs were isolated from blood donors by Ficoll-Hypaque centrifugation, and stimulated for 2–3 days with phytohaemagglutinin (5 μ g ml⁻¹) and IL-2 (100 U ml⁻¹) (Roche). CD4⁺ T cells were purified from the activated PBMCs by positive selection using anti-CD4 immunomagnetic beads (DynaL Inc.). The purified lymphocytes were cultured for at least 3 days at 2×10^6 ml in medium containing IL-2 (200 U ml⁻¹) before being used in the ¹²⁵I-MIP-1 β binding assay. The cells were screened for CCR-5-defective alleles¹⁴, and only cells from wild-type donors were used (except when specified). 293 cells were transfected with pcDNA3.1-CCR-5 (ref. 1) using the calcium phosphate method, and resistant clones were selected in culture medium containing 1 μ g ml⁻¹ neomycin (G418; Sigma). Resistant cells were subcloned and tested for CCR-5 expression in a binding assay using ¹²⁵I-MIP-1 β .

MIP-1 β binding assay and gp120 competition. Purified CD4⁺ T cells were washed twice in ice-cold binding buffer (RPMI 1640 medium containing 1% BSA, 25 mM HEPES, 0.05% sodium azide). Duplicate samples (2×10^6 cells in 200 μ l) were incubated with 0.1 nM ¹²⁵I-MIP-1 β (2,200 Ci mmol⁻¹; 0.25 μ Ci ml⁻¹) for 2 h on ice. Unlabelled ligand or gp120 (mixed with monoclonal antibodies when appropriate) was added to the cells immediately before radiolabelled ligand was added. Anti-CD4 monoclonal antibodies were added to the cells simultaneously with gp120. These cells were then separated from unbound ligand by centrifugation (60 s, 14,000g) through oil (80% silicone oil, Aldrich; 20% mineral oil, Sigma), and the radioactivity in the cell pellet was determined by gamma counting. Specific binding of ¹²⁵I-MIP-1 β was estimated by including a 100-fold excess of unlabelled MIP-1 β . Each experiment was repeated at least twice using cells from different donors. For experiments with 293-CCR-5 cells, the cells were detached with 1 mM EDTA then washed twice with binding buffer. Samples (5×10^5 cells) were incubated with 0.5 nM ¹²⁵I-MIP-1 β , then processed as above. When the ¹²⁵I-MIP-1 β concentration was reduced to 0.1 nM, no specific binding was detected.

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Crystal structure of a T-cell receptor β -chain complexed with a superantigen

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SUPERANTIGENS (SAGs) are viral or bacterial proteins that act as potent T-cell stimulants and have been implicated in a number of human diseases, including toxic shock syndrome^{1,2}, diabetes mellitus³ and multiple sclerosis⁴. The interaction of SAGs with the T-cell receptor (TCR) and major histocompatibility complex (MHC) proteins results in the stimulation of a disproportionately large fraction of the T-cell population². We report here the crystal structures of the β -chain of a TCR complexed with the *Staphylococcus aureus* enterotoxins C2 and C3 (SEC2, SEC3). These enterotoxins, which cause both toxic shock and food poisoning, bind in an identical way to the TCR β -chain. The complementarity-determining region 2 (CDR2) of the β -chain and, to lesser extents, CDR1 and hypervariable region 4 (HV4), bind in a cleft between the two domains of the SAGs. Thus, there is considerable overlap between the SAG-binding site and the peptide/MHC-binding sites of the TCR. A model of a TCR–SAG–MHC complex constructed from the crystal structures of (1) the β -chain–SEC3 complex, (2) a complex between staphylococcal enterotoxin B (SEB) and an MHC molecule⁵, and (3) a TCR V α domain⁶, reveals that the SAG acts as a wedge between the TCR and MHC to displace the antigenic peptide away from the TCR combining site. In this way, the SAG is able to circumvent the normal mechanism for T-cell activation by specific peptide/MHC complexes.

We present the crystal structures, at 3.5 Å resolution, of the bacterial SAGs SEC2 and SEC3 each bound to the extracellular portion of the β -chain (V β 8.2J β 2.1C β 1) of a mouse TCR (designated 14.3.d) specific for a haemagglutinin peptide of influenza virus in the context of I–E^d (Table 1; Fig. 1). The crystal structures of uncomplexed 14.3.d β -chain^{7,8}, SEC2 (ref. 9), and SEC3 (ref. 10) are known and we have shown that the unpaired β -chain fully retains the SAG-binding activity of the assembled $\alpha\beta$ TCR heterodimer¹¹. SEC2 and SEC3 interact identically with the TCR β -chain and none of the four amino-acid differences between SEC2 and SEC3 are located in the complex interface. This is reflected in the dissociation constants (K_D s) of the two SAGs for the β -chain, 7.9×10^{-6} M for SEC2 and 9.2×10^{-6} M for SEC3 (ref. 11), which are the same within experimental error. Superposition of the two complexes gives a root-mean-square (r.m.s.) difference of 0.46 Å for all 476 α -carbon atoms. Because of the similarity of the complexes, all subsequent discussion is limited to the TCR β –SEC3 crystal structure, but the major conclusions apply equally well to the interaction of the β -chain with SEC2. The complex is formed through contacts between the V β domain and the small and large domains of SEC3 (Fig. 1b, c). The solvent-excluded area of the interface is 1,300 Å², which is similar in size to antigen–

TABLE 1 Crystal structure determination of TCR β –SEC2 and TCR β –SEC3 complexes

	β -SEC3	β -SEC2
Data processing		
Space group	P2 ₁	P2 ₁
Cell parameters		
a (Å)	82.4	81.7
b	87.6	86.0
c	71.4	70.7
β (°)	93.3	93.2
Number of crystals	2	3
Observations to 3.5 Å	57,553	80,127
Unique reflections	11,314	9,175
Completeness (%)	89.1 (81.3)	73.3 (47.8)
I/ σ	6.8 (3.7)	4.7 (1.5)
R _{merge} (%)	12.5 (25.9)	13.7 (45.1)
Refinement		
Data range (Å)	8.0–3.5	10–3.5
Reflections ($F > \sigma F$)	9,604	7,650
Completeness (%)	82.8	64.1
Non-hydrogen atoms	3,770	3,808
R.m.s.d. bond lengths (Å)	0.008	0.006
R.m.s.d. bond angles (°)	1.7	1.5
Reflections in R _{free} set	1,157	602
R _{free} (%)	32.8	36.1
R _{work} (%)	23.8	26.2
Mean B factors (Å ²)		
V β , C β , SEC	21, 33, 35	18, 41, 37

Values in parentheses correspond to the highest-resolution shell (3.56–3.50 Å).

antibody interfaces. The V β residues in contact with SEC3 are: Asn 28 and Asn 30 of CDR1; Tyr 50, Gly 51, Ala 52, Gly 53, Ser 54 and Thr 55 of CDR2; Lys 57 and Lys 66 of framework region 3 (FR3); and Pro 70 and Gln 72 of HV4 (numbered as in ref. 8; Fig. 2a). The CDR1, CDR2, FR3 and HV4 regions account for 30, 47, 13 and 10%, respectively, of the total contacts to the SAG. However, CDR1 and Gln 72 can only potentially contact part of the SEC3 disulphide loop (residues Gly 102 to Gly 106) which is not visible in electron density. The disulphide loop is present in the 1.9 Å crystal structure of uncomplexed SEC3 but with high B-factors, indicating flexibility. It has been included in the crystal structure of the complex with atomic occupancies of zero (see Methods). There are no direct contacts between CDR3 and SEC3. The SEC3 residues in contact with V β are: Asn 60, Tyr 90, Val 91, Gly 102, Lys 103, Val 104, Gly 106 (small domain); and Gly 19, Thr 20, Asn 23, Tyr 26, Phe 176 and Gln 210 (large domain).

The crystal structure of the β -SEC3 complex readily accounts for mutational and genetic evidence implicating residues in V β CDR1, CDR2 and HV4 in SAG recognition^{2,12,13}, because these are the regions that form the interface with SEC3 (Figures 1c and 2a). On the other hand, it has been found that bacterial and viral SAGs stimulate T cells bearing particular V β elements without obvious selection for V β CDR3 length or amino-acid sequence^{2,13}. This is explained by the absence of direct contacts between CDR3 and SEC3 in the crystal structure. However, indirect effects of V β CDR3 on SAG reactivity cannot be excluded, as discussed below.

Examination of the TCR β –SEC3 complex reveals the structural basis for the similar V β specificities observed for SEB, SEC1, SEC2 and SEC3. Mutations in SEB and SEC have been identified at positions 23, 60 and 61 that affect T-cell stimulation but not MHC class II binding, suggesting specific contacts to the TCR^{2,13}. These mutations are located in a cleft between the small and large domains of these SAGs^{9,10,14} which forms the interface with the β -chain in the crystal structure (Fig. 1b). The three SEC subtypes and SEB share similar V β specificities². However, SEC1 and SEB react with human V β 3 but not V β 13.1, whereas SEC2 and SEC3 react well with V β 13.1 but only weakly with V β 3 (ref. 15). Site-directed mutagenesis has localized these reactivity differences to position 26, which is valine in SEC1 and SEB and tyrosine in SEC2

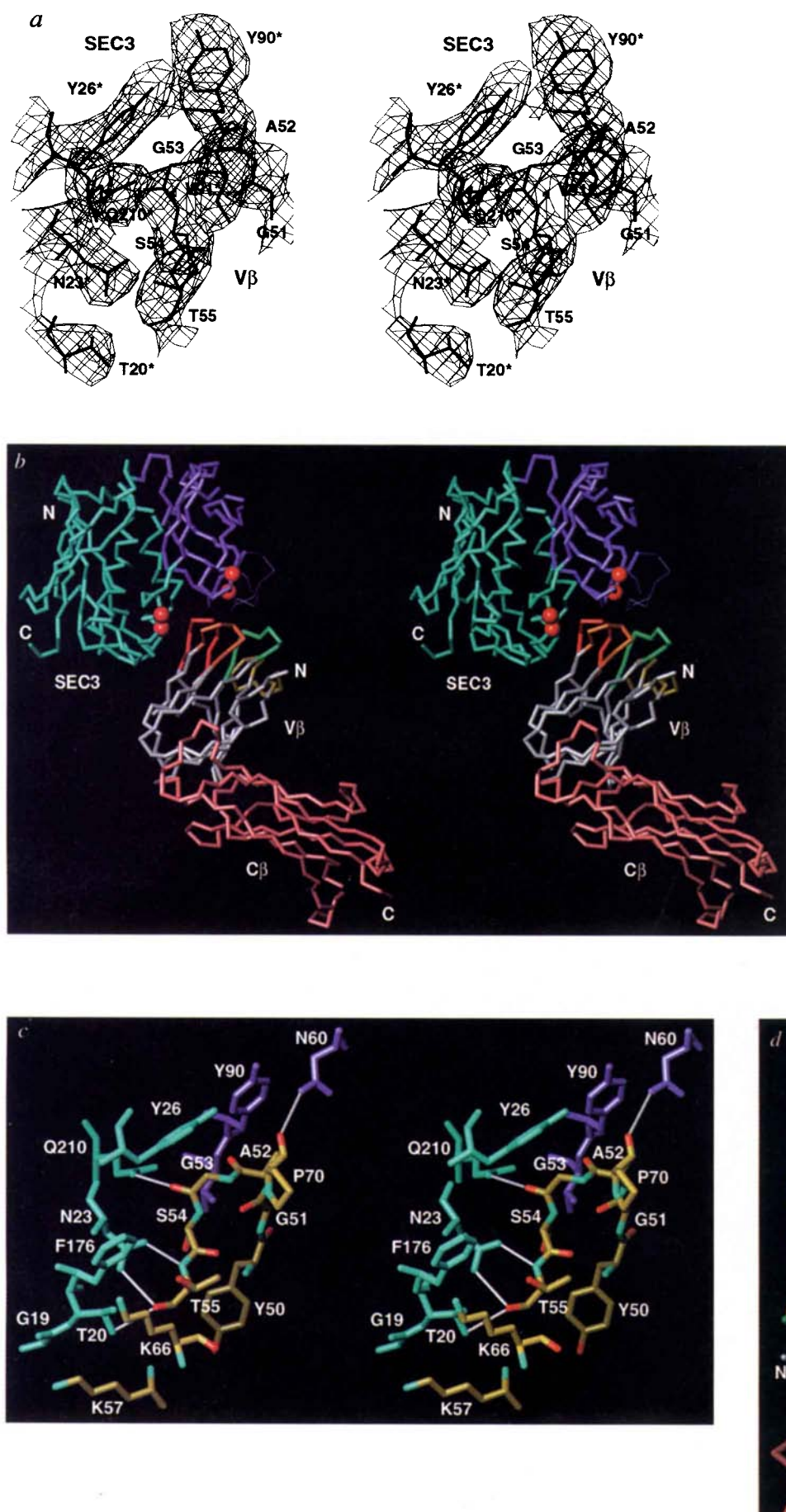


FIG. 1 TCR β -SEC3 complex. *a*, Electron density (stereo view) from the final $3.5 \text{ \AA}$ $2F_o - F_c$ map, contoured at 1σ , in the region of the TCR β -SEC3 interface. SEC3 residues are indicated with an asterisk. *b*, α -Carbon stereo view of the complex. Colours are as follows: V β (white), CDR1 (green), CDR2 (red), CDR3 (yellow), HV4 (orange), C β (pink), SEC3 large domain (blue) and small domain (purple). SEC residues that have been implicated in interactions with the TCR are drawn as spheres. The SEC3 disulphide loop was modelled according to the uncomplexed SEC3 crystal structure and is drawn with thin lines. *c*, Interactions in the V β C β -SEC3 interface. V β atoms are coloured according to atom type and SEC3 atoms are coloured purple (small domain) and blue (large domain) and hydrogen bonds are drawn as white lines. *d*, α -Carbon view with potential N-linked glycosylation sites shown as spheres. View is approximately 120° away from *b* about the vertical axis. The four potential glycosylation sites in the interface (residues 53, 57, 66, 72) are shown in white. Positions 24 and 74, which may be used by murine V β 8.2 but are not in the interface, are coloured blue. The remaining potential glycosylation sites are coloured yellow.

and SEC3. Tyrosine 26 contacts V β residue Gly 53 in the β -SEC3 complex (Fig. 2a). The identification of a single residue responsible for the difference in specificity between SEB and SEC2/SEC3 toward V β 3 and V β 13.1 implies that the disulphide loop of SEC3, which differs considerably in sequence between SEB and SEC at positions 97–107 (Fig. 2b), is not critical to V β binding. The weak binding of SEB to the 14.3.d β -chain ($K_D = 1.4 \times 10^{-4}$ M) compared to SEC1, 2 or 3 (ref. 11) can be at least partially attributed to the substitution of a tyrosine at V β -contacting position 91 in place of the valine present in all three SEC subtypes (Fig. 2b). However, differences in the disulphide loop may also contribute. It is apparent from a sequence alignment of bacterial SAGs (Fig. 2b) that staphylococcal enterotoxins A, D and E, which do not activate V β 8.2-bearing T cells², differ from SEB and SEC at nearly all V β -contacting positions. On the other hand, streptococcal pyrogenic exotoxin A (SPEA), which binds the 14.3.d β -chain with $K_D = 6.2 \times 10^{-6}$ M (ref. 11), retains several key contacting residues, in particular Asn 60, Tyr 90 and Gln 210 (Fig. 2a, b).

The crystal structure enables rationalization of data showing that SEC3 can stimulate T cells expressing V β domains from a number of different families². All of the hydrogen bonds between SEC3 and V β are formed between SEC3 side chains and V β

backbone atoms (Figs 1c and 2a). On the basis of amino-acid sequence comparisons, the positions of these backbone atoms are most likely to be the same in the relevant V β domains^{7,8}. Such a recognition mechanism, which is conformation-dependent but can be highly sequence-independent, is likely to be used by other SAGs. A sequence alignment of mouse and human V β families reactive with SEC3 illustrates the diversity of amino acids that can be accommodated at SAG-contacting positions (Fig. 2c). A similar mode of binding, involving a major role for backbone hydrogen bonds, has been observed in peptide-MHC interactions¹⁰. Of course, interactions involving V β side chains may contribute to the binding energetics but these interactions would be sequence-dependent.

No major conformational changes are observed in either the 14.3.d β -chain^{7,8} or SEC3 (ref. 10) upon complex formation. In particular, the close association between the V β and C β domains found in the structure of the free β -chain is preserved; this indicates that the quaternary structure of the β -chain is independent of crystal packing and may be relatively rigid. The unbound and SEC3-bound TCR β -chains superpose with a r.m.s. difference of 0.38 Å for all 241 α -carbon atoms. Likewise, the free and complexed SEC3 molecules superpose with a r.m.s.

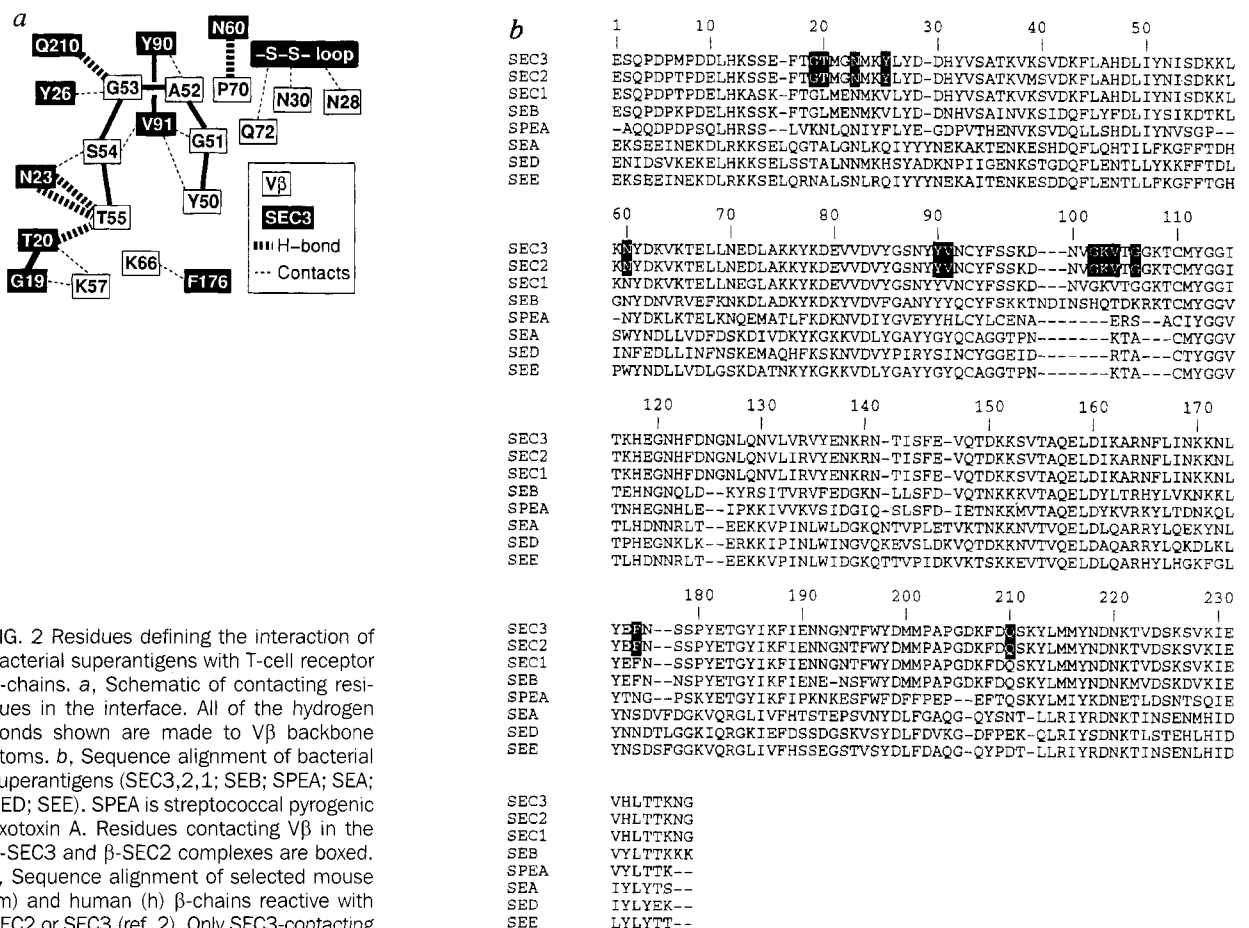


FIG. 2 Residues defining the interaction of bacterial superantigens with T-cell receptor β -chains. **a**, Schematic of contacting residues in the interface. All of the hydrogen bonds shown are made to V β backbone atoms. **b**, Sequence alignment of bacterial superantigens (SEC3,2,1; SEB; SPEA; SEA; SED; SEE). SPEA is streptococcal pyrogenic exotoxin A. Residues contacting V β in the β -SEC3 and β -SEC2 complexes are boxed. **c**, Sequence alignment of selected mouse (m) and human (h) β -chains reactive with SEC2 or SEC3 (ref. 2). Only SEC3-contacting residues are shown.

difference of 0.42 Å for all 239 α -carbons. Similarly, no large conformational changes were noted upon formation of the complex between HLA-DR1 and SEB (ref. 5). Therefore, excluding possible cooperative effects, these results suggest that formation of the TCR-SAg-MHC complex does not involve major domain movements or rearrangements in the polypeptide backbones of the interacting species.

An examination of the potential glycosylation sites on V β suggests that glycosylation may be used to modulate TCR-SAg interactions. We have demonstrated¹¹ that glycosylation does not affect the affinity of the 14.3.d β -chain for SEC1, 2 or 3. As shown in Fig. 1d, positions 24 and 74, the N-linked glycosylation sites of this particular V β , are not implicated in the β -SEC3 interface. However, four of the 17 potential N-linked glycosylation sites of human and mouse V β sequences¹⁷ are located in the binding site for SEC3. This indicates that TCR β -chains may potentially use glycosylation as a means of preventing T-cell activation by staphylococcal enterotoxins and its resulting deleterious effects on the host. However, there is likely to be considerable overlap between the binding sites for SAg and for peptide/MHC (see below), which also probably uses CDR1, CDR2 and, perhaps, HV4 (ref. 8). Introduction of a carbohydrate moiety to block the binding of bacterial SAg could therefore also abolish peptide/MHC recognition, rendering the TCR non-functional. Elimination of the glycosylation site at V β 8.2 Asn 24 by mutation to glutamate² or histidine¹⁸ has been shown to confer reactivity to Mls-1, a SAg encoded by a mouse mammary tumour retrovirus. This suggests that glycosylation can serve as a mechanism for shielding the Mls-1 binding site on V β and, furthermore, that viral and bacterial SAg bind somewhat different regions of the V β domain^{2,18}. The latter conclusion is also supported by the finding² that reactivity to Mls-1 is affected by mutations at V β positions 19 and 20, which are not part of the interface with SEC3.

Several models for T-cell activation by SAg have been proposed^{2,19}. These models differ in the extent to which the TCR interacts with the MHC in the TCR-SAg-MHC complex. The available crystal structures allow us to discriminate among these models, at least for the staphylococcal enterotoxins. We have constructed a hypothetical model of the TCR-SAg-MHC complex by least-squares superposition of the following crystal structures: (1) the TCR β -chain-SEC3 complex; (2) the SEB-peptide/HLA-DR1 complex⁵; and (3) a TCR V α homodimer⁶ (Fig. 3a). The accuracy of this model depends on the assumptions that (1) there are no major conformational changes in any of the individual components upon complex formation, (2) SEB and SEC3 bind similarly to the TCR and MHC molecules, (3) human DR and mouse I-E MHC class II molecules have similar three-dimensional structures, and (4) the geometry of V α V β association in the TCR heterodimer resembles that of the V α homodimer. The first assumption is probably valid as discussed above. The second assumption is supported by the high degree of sequence homology of these SAg (65% identical; Fig. 2b), their very similar three-dimensional structures^{9,10,14} including the sites for binding TCR and MHC, and various mutagenesis and genetic studies^{2,13}. The third assumption is validated by a comparison of the crystal structures of HLA-DR1 and I-E^k, showing nearly identical polypeptide conformations, including the peptide-binding α -helical regions²⁰. The fourth assumption is also likely to be valid since many of the amino acids forming contacts in the V α -V α interface are also present in V β (ref. 6).

Our model has several notable features. The SAg is well

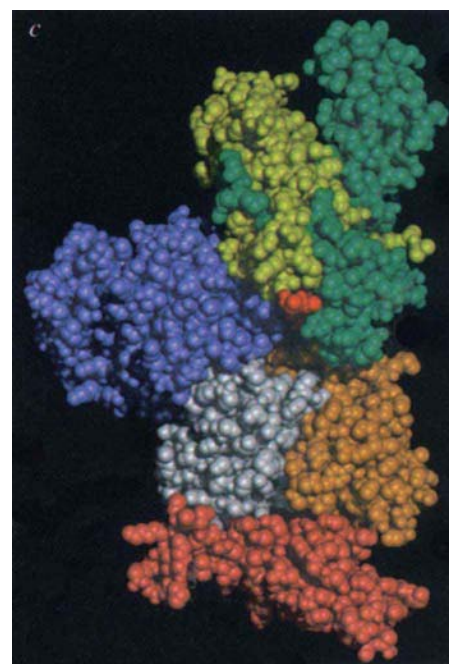
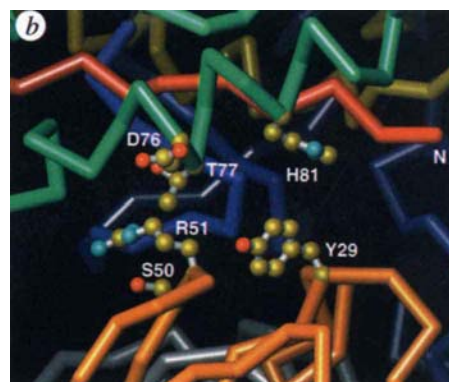
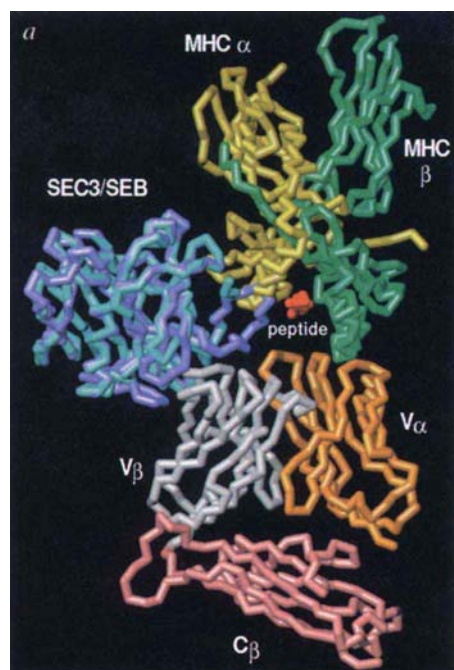


FIG. 3 Hypothetical model of TCR-SAg-MHC interactions. *a*, Superposition of V β C β -SEC3, MHC class II-SEB, and V α . The V α domain was positioned by superposing one V α domain from the V α V α crystal structure onto the V β domain. SEC3 is coloured purple and SEB blue. *b*, Close-up of putative contacts between the β -chain of class II (Asp 76, Thr 77, His 81) and the α -chain of the TCR (Tyr 29, Ser 50, Arg 51). View is $\sim 90^\circ$ from *a* about the vertical axis. The N terminus of the antigenic peptide is labelled. Human DR1 and mouse I-E class II molecules have identical amino acids at positions 76, 77 and 81. *c*, Space-filling model of V β C β V α -SEC3-peptide/MHC complex.

positioned to bridge the antigen-presenting cell and the T cell (Fig. 3a, c). The V α domain makes no direct contacts with the SAg, consistent with the ability of the β -chain alone to bind SAg^{21,13}. The MHC molecule is oriented with its α -chain over the β -chain of the TCR and its β -chain over the TCR α -chain. However, there are no direct contacts between the TCR β -chain and the MHC α - or β -chain. On the other hand, the MHC β 1 domain interacts with the V α domain of the TCR. This can account for evidence of a functional interaction between the class II β -chain and the TCR α -chain in recognizing bacterial SAg^{21,22}, as well as for evidence that certain V α regions interact more favourably than others as indicated by the preferential expression of these V α s among SAg-reactive T cells^{13,22}. In particular, residues 76, 77 and 81 of the β 1 domain α -helix of the MHC molecule HLA-DR1 are predicted to interact with V α CDR1 residue 29 and CDR2 residues 50 and 51, with no contacts to V α CDR3 (Fig. 3b). Indeed, mutations at positions 77 and 81 of the HLA-DR1 β -chain and at position 77 of the I-E^k β -chain have been found to greatly reduce the T-cell response to bacterial SAg without affecting binding of the SAg to the MHC class II molecules^{21,22}, suggesting contacts to the TCR.

The MHC molecule is only partially engaged by the TCR in our model of the TCR–SAg–MHC complex, with the SAg acting as a wedge between the TCR β -chain and the MHC α -chain. As a consequence, the antigenic peptide is largely removed from the combining site of the TCR (Fig. 3a, e). By this means, the SAg is able to circumvent the normal mechanism for T-cell activation by specific peptide/MHC complexes. This can explain the finding that formation of a TCR–SEB–DR1 complex was not affected by the presence of several different DR1-bound peptides²³. However, depending on the particular peptide bound by the MHC class II molecule, and on the conformations of the V α and V β CDR3 loops, the peptide may be able to make a small number of contacts with the TCR and so modulate SAg activity. Thus, the overall stability of the TCR–SAg–MHC complex is determined by the combined strengths of several different sets of interactions: TCR β -chain–SAg, SAg–MHC α -chain, MHC β -chain–TCR α -chain, and (possibly) peptide–TCR. In the case of the staphylococcal SAg toxic shock syndrome toxin-1 (TSST-1) bound to HLA-DR1, the SAg reaches across the antigen-binding groove such that peptide–SAg and SAg–MHC β -chain interactions may also play a role²⁴. Furthermore, in contrast to the staphylococcal enterotoxins, the mode of binding of TSST-1 to DR1 would probably also preclude direct TCR–MHC interactions. This may represent an extreme example of our model for T-cell activation by bacterial SAg and illustrates how variations in the structure and positioning of the SAg wedge provide a means for different SAg to modulate the degree of TCR–MHC interactions in the TCR–SAg–MHC complex.

Note added in proof: The recently published crystal structure of an associated $\alpha\beta$ TCR heterodimer (accession number 1TCR)³² confirms that (1) the close association between V β and C β domains observed in the free β -chain and in the β -SEC3 complex is maintained in the $\alpha\beta$ heterodimer, and (2) the geometry of V α V β association in the TCR heterodimer is very similar to that of the V α homodimer used to model the TCR–SAg–MHC complex in Fig. 3. □

Methods

Soluble, unglycosylated 14.3.d TCR β -chain was obtained by elimination of potential N-linked glycosylation sites through site-directed mutagenesis and introduction of a termination codon at the end of the first C-region exon⁷. The truncated β -chain was produced in J558L myeloma cells and affinity-purified using an anti-C β monoclonal antibody. Further purification was carried out on a Pharmacia Mono-S cation-exchange column equilibrated with 50 mM MES, pH 6.5, and developed using a linear NaCl gradient. Recombinant SEC3 from *Staphylococcus aureus* strain FRI913 was prepared as described²⁵ and further purified using a Pharmacia Mono-Q anion-exchange column equilibrated with 20 mM Tris–HCl, pH 8.5; the protein was eluted using a NaCl gradient. For crystallization, the V β C β chain and SEC3 were mixed in an equimolar ratio and dialysed against 20 mM Tris–HCl, 25 mM NaCl, pH 7.5. Crystals were grown at room temperature in hanging drops by mixing 2 μ l of a solution containing 19 to

21% PEG1000, 0.1 M MgCl₂, 0.025% agarose, 0.1 M Tris–HCl, pH 6.5, with an equal volume of protein solution at 10 mg ml⁻¹; equilibration was against a reservoir solution of the same buffer without agarose. The crystals contain two complexes in the asymmetric unit. X-ray diffraction data were recorded at 5 °C from two crystals at beamline 7-1 of the Stanford Synchrotron Radiation Laboratory using a MarResearch Imaging Plate detector. Using the HKL/Denzo/Scalepack package²⁶, 57,553 observations were reduced to 11,314 unique reflections with $R_{\text{merge}} = 12.5\%$ (completeness 89.1% to 3.5 Å). The structure was solved using the molecular replacement method with the program AMoRe (ref. 27). Search models consisted of the 14.3.d TCR β -chain (refined at 1.7 Å resolution)⁷ and SEC3 (refined at 1.9 Å resolution)¹⁰. Unambiguous solutions were found in the cross-rotation and translation functions for both complexes in the asymmetric unit. Refinement was carried out using X-PLOR (ref. 28) using strict non-crystallographic symmetry (NCS) constraints²⁹. The use of strict NCS constraints results in a twofold reduction in the number of refinable parameters in this case and enabled maximal reduction of R_{free} , the residual calculated from a set of reflections omitted from the refinement calculations. Likewise, only one B-factor per residue was refined initially whereas two B-factors per residue were refined in the final stages. R_{free} was used from the start of refinement until the final cycle when all reflections were included. Solvent-flattening and map-averaging using PHASES (ref. 30) resulted in some improvements in electron density but not for the SEC3 disulphide loop. Residues that had no electron density (TCR β residues 133–142, 220–230, 245–246; SEC3 residues 1, 96–107, 124–126, 191–193, 226, 237–239) were retained in the model because these residues were located in the crystal structures of both uncomplexed components. However, these residues were given occupancies of zero in the complex. Structure determination of the β -SEC2 complex followed similar procedures. A Ramachandran plot of the β -SEC3 complex as analysed by PROCHECK (ref. 31) indicates that 81.5, 18.3 and 0.2% of residues fall in favoured, allowed, and disallowed regions, respectively. Corresponding values for the β -SEC2 complex are 79.7, 19.6 and 0.7%.

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CORRESPONDENCE and requests for materials should be addressed to R.A.M. (e-mail: mariuzza@indigo2.carb.nist.gov).

Natural choices for the neurosciences

Various new systems and reagents for neuroscientific study are featured this week, including a new drug delivery system for therapeutic agents, a receptor available for drug screening programmes and a vibrating ion probe system.

THE new Leica TCS-NT **confocal microscope** has been designed to improved image quality with resolution at the theoretical limits by integrating the optical design of the scanner, microscope and objectives. According to the manufacturer the compact alignment-free ultraviolet/visible confocal module can be exchanged between upright and inverted microscopes by the user in less than five minutes. The system is designed for multi-colour experiments with shift-free multi-line illumination and flexible single- or multiple-laser illumination modules for up to five fibre-coupled ultraviolet/visible lasers and an AOTF wavelength mixer. The true illumination pinhole and a single computerized detection pinhole are designed to assure beam geometry. Moreover, the system can be equipped with up to five simultaneous detectors



Leica's TCS-NT confocal microscope.

along with an internal cooling option. The software provides full three-channel 24-bit, three-dimensional reconstruction and animation with multiple rendering modes supporting the visualization of acquired data. One of the features is live optical cross-sectioning with improved precision and speed owing to a focusing stage. The system uses a standard PC workstation with the widely compatible multitasking operating system Windows NT. A high numerical aperture, long working distance and water immersion objectives should provide quality imaging results, even with thick specimens.

Reader Enquiry No. 100

Packard Instruments recently introduced the Cyclone **storage phosphor system for filmless autoradiography** of radiolabelled gels, blots and tissue sections. The system features a variety of reusable, high-performance storage phosphor screens to suit a variety of applications. The multi-



Cyclone phosphor storage system.

purpose (MP) screens are specially coated for moisture resistance and durability for typical gels and blots. The 'ST' screens are thicker and formulated to be up to twice as sensitive as typical storage phosphor screens for low activity ^{32}P and ^{125}I applications. The SR screens are formulated from the finest grain of phosphor crystals to provide the best resolving power for high resolution ^{35}S , ^{33}P and ^{14}C applications. The 'TR' tritium sensitive screens are like the 'SR' screen, only they are uncoated so that it can detect the low-energy ^3H in tissue sections at higher resolution. The screens are available in three sizes to accommodate different types of samples: small screens can easily fit two minigels, medium screens fit most full-sized blots and gels and the long screens cover the length of a sequencing gel.

Reader Enquiry No. 101

The Gliadel-Wafer is the first **brain cancer treatment** of its kind to be offered by Rhône-Poulenc Rorer allowing the delivery of chemotherapeutic agents directly to the tumour site. Gliadel complements the traditional therapies of surgery, radiation and intravenous chemotherapy, adding a new approach to the treatment of the most aggressive form of brain cancer. This approach consists of a biodegradable polymer wafer saturated with the chemotherapeutic drug carmustine (BCNU). Up to eight wafers are inserted into the cavity created during the surgical removal of a brain tumour. After implantation, the wafers slowly erode permitting BCNU to be delivered directly to the tumour site for a two- to three-week period. In animal studies, according to the company, approximately 62 mg of BCNU delivered locally through Gliadel provided brain-tissue concentrations of BCNU 100 to 1,000 times higher than administration

of a 3,000-mg intravenous dose. This method of delivery is designed to reduce the systemic side effects that are typically seen with the administration of intravenous chemotherapy. The wafer was cleared for marketing by the US Food and Drug Administration in September of this year for use as an adjunct to surgery in patients with recurrent glioblastoma multiforme for whom surgical resection is indicated. It is formulated as 200-mg round disk wafers, 14 mm in diameter and 1 mm thick.

Reader Enquiry No. 102

Imaging systems

Kinetic Imaging, together with AstroCam, offers a high-performance ratio imaging system for the neurosciences in the area of low-light imaging and ratio fluorescence. Kinetic has adapted the Calcium PC the ratio fluorescence system to operate with the Capella 4100 high-speed cooled CCD camera. Designed for intracellular ion imaging, Calcium PC offers rapid capture of 8-, 12- or 16-bit images (up to 25 frames per second), fully integrated control and true multidimensional imaging. With floating point precision and through-series

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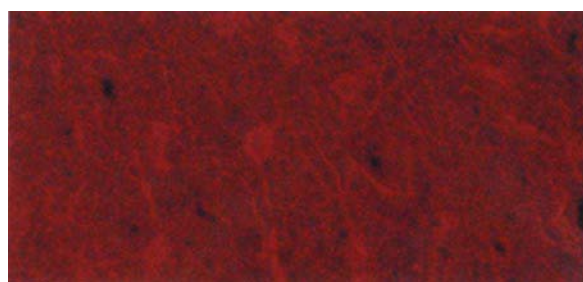
analysis, Calcium PC offers benefits to researchers in the neurosciences, including DAMM (disk aware memory management) system, which provides multi-gigabyte, on-line image memory using direct control of SCSI AV disk drives, thereby allowing open-ended acquisition of dynamic image data. The Capella from AstroCam is a high-speed, cooled CCD camera (up to 8 MHz) with 12-bit precision, offering high stability and sensitivity, as well as windowing and binning capabilities. CCD options include Kodak 768 × 512 and 1317 × 1035 pixel sensors. However, the company recommends the 512 × 512 frame transfer device for high speed performance imaging.

Reader Enquiry No. 103

Hamamatsu has released a new version of its Argus-50/Ca²⁺ intracellular calcium analysis software for **calcium and other ion imaging**, a technique that is useful for investigating cell physiology. This improved version allows up to 100 locations on the image to be monitored for kinetic changes. These regions can be of rectangular, elliptical or arbitrary shape,

allowing sample regions to be more accurately defined. In addition, there is an option now offered that allows the simultaneous measurement of two fluorescent probes within a single cell. This allows the researcher to investigate the Ca²⁺ concentration and pH, as well as other ions or intracellular macromolecules, such as proteins that have undergone fluorescence tracing within a cell. Also being launched is a new high frame rate cooled CCD cameras designed for fluorescence and ration imaging. The system should find use with PCs aimed at the microscopy and biological sciences fields. It is designed to be a highly flexible system that can be configured for a wide range of applications with either video or high-resolution digital image acquisition. Various options are available where photon counting techniques are employed, including general fluorescence, Ca²⁺ ion imaging, FISH and luminescent imaging.

Reader Enquiry No. 104



Chemicon's goat polyclonal antiserum to vesicular acetylcholine transporter for use in immunohistochemistry.

immunohistochemistry on rat and mouse tissue. It labels cholinergic cell bodies in the septum and nucleus basalis, as well as cholinergic fibres in the brain stem and spinal cord. These properties should make it a useful tool for Alzheimer research, as acetylcholine is one of the first transmitters to be reduced in this disease. Also, researchers in neuromuscular diseases or in the autonomic nervous system should find this valuable in their research.

Reader Enquiry No. 106

Zymed Laboratories has manufactured a new **monoclonal mouse anti-neuron-specific enolase (NSE) antibody** that is formulated for staining frozen or formalin-fixed, paraffin-embedded tissue sections. NSE is a cytoplasmic protein expressed in neuronal cells and neuronally derived tumours and may be useful for the demonstration of peripheral neuroendocrine tumours, for example neuroblastoma or lung small-cell carcinoma. Upon histological staining, NSE displays diffuse cytoplasmic staining in positive cells. The company states that its anti-NSE monoclonal antibody (clone NSE-1G4) does not cause background staining associated with some polyclonal antibodies and that it is said to be more sensitive than many monoclonal antibodies. The NSE-1G4 clone is available as a concentrate or in two different ready-to-use formulations: first generation predilute or as a second generation predilute that has been optimized for use with Histostain-Plus and HistoST5050 systems. The second generation formulation contains a protein blocker that should eliminate the need for a serum blocking step when immunostaining.

Reader Enquiry No. 107

Affiniti has released a new **polyclonal antiserum to neurogranin**, also known as the B-50 immunoreactive C-kinase substrate (BICKS), p17 and RC3. This rabbit antibody is supplied in purified form and exhibits reactivity against neurogranin in a range of mammalian species. Raised against a synthetic peptide derived from the rat molecular form of neurogranin (amino acid sequence rat NRG [9-25]), the antiserum is suitable for use in western blotting, immunoprecipitation and immunohistochemistry at dilutions of up

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READER ENQUIRY NO. 61

Antibodies and antiserum

Wako's **anti-human nerve growth factor (anti-hNGF) antibody**, designated clone NGFA133, is a prominent neuronal presence necessary for differentiation and up keep of sensory and sympathetic peripheral nervous system function, along with various brain neuronal functions. This antibody is for characterizing NGF neutralization activity, as well as ELISA assays that benefit from its homodimer binding formation. The monoclonal anti-hNGF antibody is produced by hybridoma clone NGFA133, established by fusing myeloma cells P3U1 and spleen cells of BALB/c mice, which is immunized with recombinant hNGF. The antibody is purified by protein A affinity chromatography from ascites fluid of BALB/c mice and prepared in PBS solution, no preservative or stabilizer are used. This item is not cross-reactive with human NT-3 nor SDS-denatured hNGF, and is intended for research use only, according to the manufacturer.

Reader Enquiry No. 105

Chemicon now offers its new goat polyclonal **antibody to vesicular acetylcholine transporter (VACHT)**. The antibody is stated to have been used successfully for



Wako's anti-hNGF.

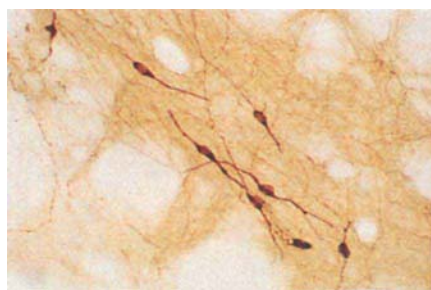
to 1:1,000. The manufacturer states that it is useful in a variety of mammalian species, including rat, goat and cow. The peptide used to raise the antiserum is available for use in solid-phase assays (for example, ELISA) and for liquid-phase applications, such as a standard, as a ligand and suitable for radiolabelling and as an antibody adsorption control.

Reader Enquiry No. 108

Dako's clone NGFR5 monoclonal antibody against nerve growth factor receptor (NGFR) recognizes the 75K NGF receptor p75^{NGFR}. The p75 receptor protein is a member of the nerve growth factor/tumour necrosis factor superfamily of receptors, many of which function as mediators of cell death or apoptosis. *In vitro*, unbound p75^{NGFR} has been demonstrated to promote neural cell death, whereas binding of p75^{NGFR} by NGF ligand or antibody has been shown to inhibit p75^{NGFR} induced cell death. Immunohistochemical analysis with this antibody can be performed routinely on processed tissue sections, frozen section or cell smears. Other apoptosis-related antibodies are also available: bcl-2 (M0887), p53 (M7001), Lewis^y (M4504) and NGFR (M3507).

Reader Enquiry No. 109

Incstar has announced the release of a rabbit polyclonal antiserum to brain nitric oxide synthase (NOS) for immunohistochemistry. The NOS enzyme is responsible for the synthesis of nitric oxide (NO) by oxidation of arginine. Three forms of NOS have been identified; the two constitutive forms and one inducible form are brain NOS (bNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), respectively. In addition, three isotypes of bNOS (alpha, beta and gamma) have been recently discovered. The bNOS protein is approximately 155K and is found mainly in neural tissues. This anti-bNOS recognizes all three isotypes of the enzyme. The antiserum was generated in rabbit against a C-terminal synthetic peptide from human bNOS. Immunostaining is completely abolished by pre-adsorption with 5 µg of peptide per ml of diluted bNOS antiserum. In western blot analysis of brain homogenates the antiserum specifically detects a single band of approximately 155K representing bNOS. Using immunohistochemical methods and western blot, no cross-reactivity with eNOS or iNOS is observed. According to the manufacturer, anti-bNOS has been successfully used to label bNOS in

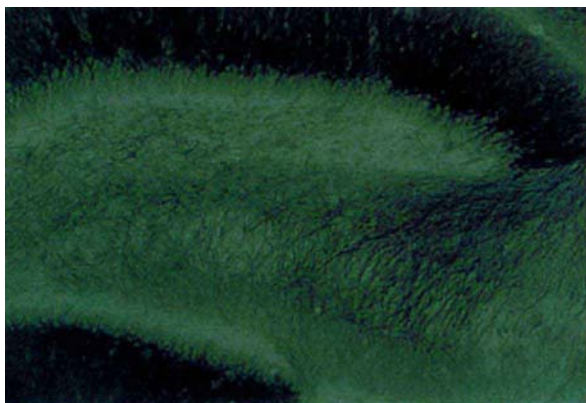


Incstar's immunohistochemistry of bNOS.

human, rat, mouse, cat, guinea pig and monkey tissues.

Reader Enquiry No. 110

Sigma BioSciences offers a diverse range of neuroscience products and signal transduction antibodies for research, including monoclonal and polyclonal antibodies. Performance is evaluated in most cases by immunoblotting (western) and immunohistochemical localization of proteins in tissue sections and cultured cells. Monoclonal antibodies are supplied as delipidized, filtered ascites fluid, unless otherwise indicated. Polyclonal antibodies



Sigma BioSciences' products for neuroscience research.

are pooled delipidized whole antisera, unless otherwise indicated. Most products are supplied as liquid, containing 0.1 per cent sodium azide, except antibodies to growth factors, which undergo filtered at 0.2 µm with no preservative added.

Reader Enquiry No. 111

Odds and ends

BioResearch Ireland now offers a cloned 5HT_{5A} receptor, available for use in drug-screening programmes that assessing the effects of compounds on the nervous system. This receptor is designed for use in research on neuroreceptors of therapeutic interest. Such receptors are now commonly used in programmes to screen compounds that have neuroactive drug potential. The 5HT_{5A} receptor cDNA clone available from the company is for a human receptor belonging to the G-protein-coupled 7-transmembrane receptor superfamily.

The receptor has been cloned into two plasmids: pBS-h5HTR_{5A} for use in generating an antisense probe for northern blot analysis, RNase protection analysis or *in situ* hybridization; and pBKCMV-h5HTR_{5A} for use in expression studies. The latter plasmid, containing the clone, can be transfected into a suitable cell line (for example, HEK 293 cells) where both transient and stable expression of the receptor can be achieved. It is provided in pBluescript SK II+ or pBK-CMV and can be released from both plasmids using the restriction enzymes *Cla*I and *Kpn*I.

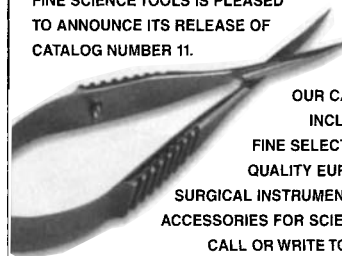
Reader Enquiry No. 112

Cellpath has introduced Neuroslide 21, designed to be a simple solution to the problem of storing larger sized microscope slides. Designed with the needs of the neuropathologist in mind, this product uses a plastic wallet with 21 compartments to enable a variety of slide sizes to be held. There are nine 76 mm × 76 mm pockets, six 50 mm × 76 mm pockets and six 26 mm × 76 mm pockets. Moreover, the 76 mm × 76 mm pockets can be used to store 35 mm transparencies. The

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PRODUCT REVIEW

Neuroslide 21 wallet is supplied with a suspension bar for use in any conventional filing cabinet and allows rapid retrieval of slides, irrespective of reviewing location. Slides of different size but from the same case study can be kept together for rapid retrievability.

Reader Enquiry No. 113

Physiological measurement

Axon Instruments has released the Axopatch 200B, a **patch-clamp amplifier** that is stated to be an extremely low-noise picoammeter capable of measuring sub-picoampere currents with a bandwidth up to 100 kHz. This amplifier should prove useful in the study of the properties of individual ion-channel macromolecules, as well as for applications in physics and engineering. Through technical improvements, such as active cooling of headstage components, the current noise has been reduced to as low as a stated 15 fA r.m.s (0.1–10 kHz). Other improvements include a new slim headstage design, a three-current measuring range from sub-picoampere currents up to 200 nA currents. The input headstage attaches to the rack-mountable main unit through a 10-foot cable, allowing greater flexibility in positioning. Commands may be internally generated or generated with computer control; telegraph outputs communicate gain settings to the software for ease and security of data recording.

Reader Enquiry No. 114

Measurement of microscopic voltages, currents and hence ionic fluxes in biological preparations using **vibrating electric field sensing probes** is now an analytical tool that can be utilized in the Uniscan range of vibrating probe instrumentation. The company has developed a scanning vibrating probe system that allows the technique to be exploited in the study of cellular currents and electric fields generated in biological media. The main advantage of the vibrating technique is the ability to detect extremely small current densities with high spatial resolution. Currents of nanoamperes with spatial resolution of micrometers are not uncommon. The manufacturer states that this is achieved by using fine microelectrodes in conjunction with a sensitive a.c. signal recovery system, detecting the signal in phase with the reference driving a piezo-ceramic vibration actuator. This instrument is controlled exclusively through a personal computer under a Windows operating system. The instrument utilizes a high-precision x, y and z positioning and scanning mechanism to enable the vibrating probe to be positioned with micrometer accuracy or to scan over specified regions and generate two-dimensional maps of localized currents and fields. Probe position is mea-



Electrophysiological processing with Neurolog, produced by Medical Systems

sured using optical encoders on all three axes and can be verified using a long range optical microscope.

Reader Enquiry No. 115

Medical Systems has announced the release of the NeuroLog system, designed to provide flexibility to instrumentation when **processing electrophysiological signals**. The system is modular: over 40 types of interchangeable modules are offered for signal conditioning, amplification, timing, averaging, integration, and others. Only those modules required to perform a particular function need to be inserted into the mountable 48-cm case. Because the unit is designed to function as a system, input and output levels, impedances and connectors of different modules are constructed to be compatible. Problems often encountered

when interconnecting pieces of equipment of diverse origin are said to be eliminated

Reader Enquiry No. 116

Literature

Research Biochemicals, represented in the UK by Semat, offers a comprehensive line of biochemicals for neuroscience applications, including adenosines, adrenergics, anaesthetics, antibodies, dopaminergics, excitatory and inhibitory amino acids, PET and spectroscopic reagents, signal transduction agents and neuropeptides. The 1996 catalogue, *Neurochemicals for the Neuroscientist*, lists the complete range of products and is available upon request. New products just introduced include the animal anaesthetics Halothan USP, recommended for euthanasia, when only an inhalation agent is required for the experimental protocol, and ketamine and xylazine, which when co-administered by injection produce rapid and reversible anaesthesia in experimental animals. The D4 dopamine receptor antagonists L-745, 870 and U-101958 have also been added to the company's range.

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These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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